INÊS CALADO REIS

DIES1 GENE EXPRESSION AND REGULATION IN GASTRIC CANCER

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TABLE OF CONTENTS

INDEX OF FIGURES

INDEX OF TABLES

x

RESUMO

Dies1 foi recentemente descrito como um novo gene envolvido na diferenciação de *embryonic stem cells* de ratinho. Aloia et al. demonstraram que a expressão deste gene aumenta durante a diferenciação de *embryonic stem cells* em cardiomiócitos e astrócitos e durante a adipogénese e neurogénese. Além do aumento da sua expressão durante a diferenciação celular, a supressão deste gene permite a manutenção das *embryonic stem cells* de ratinho num fenótipo indiferenciado. A modificação do programa de diferenciação parece ser comprometido devido a alteração da via de sinalização da BMP4 onde a proteína codificada pelo gene *Dies1* evidencia uma função de coreceptor. Além do envolvimento deste gene na diferenciação de *embryonic stem cells,* resultados preliminares realizados no nosso grupo (dados não publicados) revelaram que este gene poderá ter um função no programa de transição epitélio-mesênquima e no processo reverso. Em particular, o nosso grupo observou que a expressão do *Dies1* diminuía concomitante com a transdiferenciação da célula epitelial. Nestes dois processos, diferenciação das *embryonic stem cells* e transição epitélio-mesênquima, dois mecanismos de regulação da expressão génica têm sido descritos e associados com a expressão do *Dies1*, nomeadamente a regulação por microRNA-125a (miR-125a) e miR-125b e a metilação do promotor do gene.

Uma vez que o *Dies1* se encontra associado a dois programas de diferenciação celular, colocamos a hipótese de que este gene poderia estar envolvido na diferenciação celular, não apenas no que diz respeito à diferenciação de *embryonic stem cells* e na transdiferenciação de epitélio para mesênquima, mas também em outros programas de diferenciação, como o cancro. Desta forma, o nosso objectivo é estudar o papel do *Dies1* em cancro gástrico e o possível mecanismo de regulação de expressão que pode levar à expressão diferencial deste gene.

Para alcançar este objectivo, a expressão do gene *Dies1* e o possível mecanismo de regulação foi estudado numa série de linhas celulares de cancro gástrico. Além disso, numa série de amostras de cancro gástrico foi também estudada a expressão do gene *Dies1,* assim como a expressão de um marcador epitelial (*CDH1*) e mesenquimal (*CDH2*) de forma a encontrar associações relevantes entre a expressão destes genes e do gene em estudo.

Os nossos resultados revelaram que nas linhas de cancro gástrico o gene *Dies1* tem uma menor expressão que o estômago normal. No estudo de um possível mecanismo que estivesse na origem da diminuição de expressão deste gene, sequenciamos toda a

região codificante do gene *Dies1*, no entanto nenhuma mutação foi encontrada. Foram também estudados outros mecanismos de regulação: a metilação do promotor do gene *Dies1* e a expressão do miR-125a. Nestes estudos observamos que nas linhas celulares GP202 e AGS, a ilha CpG do gene *Dies1* encontrava-se parcialmente metilada ou hipermetilada. Além destes resultados, as linhas celulares AGS, KATO III e MKN28 demonstraram uma elevada expressão do miR-125a concomitante com a diminuição dos níveis de expressão do *Dies1*. Nas restantes 3 linhas celulares de cancro gástrico estudadas, nenhuma associação foi encontrada entre a expressão do miR-125a ou o estado de metilação do promotor e a expressão do *Dies1*. Estes resultados sugerem que a presença de outros mecanismos regulatórios podem estar na origem da diminuição da expressão deste gene.

Na nossa série de cancro gástrico a expressão do gene *Dies1* foi, no geral. aumentada e apenas 6 das 25 amostras tumorais apresentaram uma diminuição da expressão do *Dies1* quando comparado com o respectivo par normal. Estes resultados foram inconsistentes com os resultados preliminares. Esta inconsistência pode ser devida ao facto de terem sido utilizados diferentes métodos para a normalização da expressão do gene (*pool* de amostras normais vs. amostras normais pareadas). Análises futuras com um maior número de casos será necessário para revelar a variação da expressão do *Dies1* em cancro gástrico. Apesar disso, nós observamos que a expressão do *Dies1* estava aumentada no cancro gástrico com uma positiva correlação com a expressão do gene *CDH1* (marcador epitelial).

Globalmente, o nosso estudo sugere que o *Dies1* encontra-se diferencialmente expresso no cancro gástrico e que diferentes mecanismos de regulação podem explicar esta diferente expressão. A correlação observada entre a expressão do gene *Dies1* e *CDH1* sugere uma associação entre o *Dies1* e uma diferenciação epitelial.

ABSTRACT

The role of Dies1 gene as a new player in mouse embryonic stem cells differentiation was recently discovered. Aloia et al. have shown that *Dies1* expression increases during differentiation of embryonic stem cells into cardiomyocytes and astrocytes and in mouse adipogenesis and neurogenesis. In addition, by supressing *Dies1* expression the differentiation program is impaired and mouse embryonic stem cells maintain their undifferentiated phenotype. The differentiated program seems to be compromised due the alteration of BMP4 pathway, where Dies1 protein seems to have a regulatory co-receptor function. Additionally to the involvement of *Dies1* gene in embryonic stem cells differentiation, preliminary results from our group (unpublished data) reveal a possible role of this gene in epithelial to mesenchymal transition and mesenchymal to epithelial transition programs. In particular, our group has observed that *Dies1* expression decreased in concomitance with the epithelial cell transdifferentiation. In these two processes, embryonic stem cells differentiation and epithelial to mesenchymal transition, two different regulatory mechanisms have been described and associated with *Dies1* expression, namely the regulation by microRNA-125a (miR-125a) and miR-125b and promoter methylation.

Due the association of *Dies1* with two different programs of cellular differentiation we hypothesize that this gene may be involved in cell differentiation not only at embryonic stem cell level, as well as in epithelial to mesenchymal transdifferentiation and could also be relevant in other differentiation programs, such as in cancer. Therefore our main aim was to dissect the role of *Dies1* in gastric cancer and its possible expression regulatory mechanisms that may lead to a differential expression of this gene.
To achieve this goal, *Dies1* expression and possible regulatory mechanisms were studied in a set of gastric cancer cell lines. In addition, *Dies1* expression was studied in a series of gastric cancer samples in concomitance with an epithelial and mesenchymal marker, in the search for relevant associations.

Our data has shown in gastric cancer cell lines screened *Dies1* was down-regulated when compared to normal stomach. In the search for possible mechanisms underlying such downregulation, we have sequenced the entire coding locus of *Dies1*, however no mutations were found. Alternative regulatory mechanism assessed were *Dies1* promoter methylation status and miR-125a expression. We observed that in AGS and GP202 cell

lines, *Dies1* CpG island was partially methylated or hypermethylated. In addition, AGS, KATO III and MKN28 cell lines displayed an elevated expression of miR-125a in concomitance with decreased *Dies1* expression levels. For the remaining 3 gastric cancer cell lines screened, no significant association with miR-125a or promoter methylation status were observed, suggesting the presence of others regulatory mechanisms that can explain the down-regulation of this gene.

In our gastric cancer series *Dies1* was an overall overexpression, and only 6 out of 25 tumour samples screened present a down-regulation of *Dies1* expression when compared to the normal pair. These results were inconsistent with the preliminary results, which can be due to different approaches used for gene expression normalization (pool of normal samples vs. paired normal samples). Further analyses with a higher number of cases will be necessary to reveal the expression variation of *Dies1* in gastric cancer. Nevertheless we observed that *Dies1* expression was increased in gastric cancer with a positive correlation with the *CDH1* expression levels (epithelial marker).

Overall, our study suggests that *Dies1* is differentially expressed in gastric cancer and different mechanisms might explain its expression. The correlation observed between *Dies1* and *CDH1* expression suggests an association of *Dies1* with epithelial differentiation.

ABBREVIATIONS

5mC	5-Methylcytosine
A	Adenine
aa	Amino acid
AKT	Protein kinase B
ALK	Activin receptor like kinase
ATCC	American Type Culture Collection
BMP	Bone morphogenetic proteins
Bp	Base pairs
C	Cytosine
CDH1	Cadherin-1 (E-cadherin)
CDH2	Cadherin-2 (N-cadherin)
cDNA	Complementary DNA
DAB	3, 3'-diaminobenzidine
Dies1	Differentiation of Embryonic Stem cell 1
EBV	Epstein-Barr vírus
ECM	Extracellular matrix
EDTA	Ethylenediaminetetraacetic
EGF	Epidermal growth factor
EGFR	Epidermal growth factor receptor
EMA	European Medicines Agency
EMT	Epithelial to Mesenchymal Transition
epiSC	Epiblast stem cell
ERK	Extracellular signal-regulated kinases
FDA	U.S. Food and Drug Administration
FGF2	Fibroblast growth factor 2
G	Guanine
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GI24	Platelet receptor GI24 precursor
H. Pylori	*Helicobacter pylori*
H&E	Heamatoxylin and eosin
HER2	Epidermal growth factor receptor 2
HGFR	Hepatocyte growth factor receptor
HRP	Horseradish peroxidase
Id	Inhibitors of Differentiation
IGF-1R	Insulin-like growth factor 1 receptor
IHC	Immunohistochemistry

JNK	JUN N-terminal kinase
KD	Knock-down
KRAS	Kirsten rat sarcoma viral oncogene homolog
LIF	Leukemia inhibitory factor
LOH	Loss of heterosygosity
mAb	Monoclonal antibody
MAPK	Mitogen-activated protein kinase
mESC	Mouse embryonic stem cell
MET	Mesenchymal to Epithelial Transition
miRNA/miR	MicroRNA
miRNP	MicroRNA ribonucleoprotein
MLH1	MutL homolog 1
MMP	Matrix metalloprotease
mRNA	Messenger RNA
MSH2	MutS protein homolog 2
MSI	Microsatellite instability
MT1-MMP	Membrane-type1 matrix metalloproteases
mTOR	Mammalian target of rapamy
NCAM-1	Neural cell adhesion molecule 1
NFκB	Nuclear factor κ-light-chain-enhancer of activated B cells
NHERF	Na+/H+ exchanger regulatory factor
PCR	Polymerase chain reaction
PD-1H	Programmed Death-1 Homolog
PDGF	Platelet-derived growth factor
PI3K	Phosphatidylinositol-4,5-bisphosphate 3-kinase
qRT-PCR	Quantitative real time PCR
RUNX3	Runt-related transcription factor3
SHH	Sonic hedgehod
shRNA	Short hairpin RNA
SISP-1	Stress-induced secreted protein-1
SNP	Single nucleotide polymorphism
SPEM	Spasmolytic-polypeptide-expressing metaplasia
T	Thymidine
TBS-T	Tris-buffer saline containing 0.02% Tween 20
TGF-β	Transforming growth factor-β
TMA	Tissue microarray
TSG	Tumour suppression genes
TSS	Transcription start site
UTR	Untranslated region
VEGF	Vascular endothelial growth factor

VEGF	Vascular endothelial growth factor
VISTA	V-domain Ig suppression of T-cell activation
WHO	World Health Organization
ZO	Zonula occludens

CHAPTER 1

GENERAL INTRODUCTION

GENERAL INTRODUCTION

The following general introduction section concerns the three main subjects studied in this thesis. Firstly, we will present the current knowledge on *Differentiation of Embryonic Stem cell 1* gene and its involvement with embryonic stem cell differentiation. Secondly, we will present the general alterations associated with epithelial mesenchymal transition and its association with cancer. A brief description of gastric cancer, its molecular features and its association with epithelial-mesenchymal transition process is the third issue that will be addressed in this chapter.

1. *Dies1*: a gene involved in cell differentiation

A new player involved in mouse embryonic stem cell (mESC) differentiation was recently discovered by Aloia, et al. (2010). This gene was recognized in a short hairpin RNA (shRNA) functional screening for genes, whose suppression, resulted in failure of mESCs undergoing an *in vitro* neural differentiation (1). This gene (riken cDNA 4632428N05) was named *Differentiation of Embryonic Stem cell 1* (*Dies1*), however is also known as *V-domain Ig suppression of T-cell activation* (*VISTA*), *Platelet receptor GI24 precursor* (*GI24*), *Programmed Death-1 Homolog* (*PD-1H*), *Stress-induced secreted protein-1* (*SISP-1*) or *chromosome 10 open reading frame 54* (*C10orf54*) (1-4).

The Dies1 protein sequence is highly conserved in numerous vertebrate species, with high similarity between mouse and human (85.6%). In humans, this gene is localized in the reverse strand at chromosome 10 (location q22.1), in the following genomic coordinates: chr10: 71,747,559-71,773,498. The expected protein encoded by this gene is composed by 311 amino acids (aa) in humans (309 aa in murine species) (5). The protein sequence predicts a transmembrane protein, with a cytoplasmic region and a hydrophobic helix with transmembrane characteristics (1). In the N-terminal region, this protein exhibits possible Asn-glycosylation sites, as do many receptor proteins, and a V-type Ig-like domain, also present in proteins with adhesion functions such as cadherins. These two characteristics support the transmembrane categorization of Dies1 (**figure 1)** (1). Its transmembrane tract is similar to single pass membrane proteins such as Cadherin 6 and Neural cell adhesion molecule 1 (NCAM-1), while its cytoplasm domain contains numerous possible serine, threonine and tyrosine residues and does not have homologies with others proteins (1). By immunocytochemical detection it was possible to confirm the transmembrane localization of Dies1 protein (5).

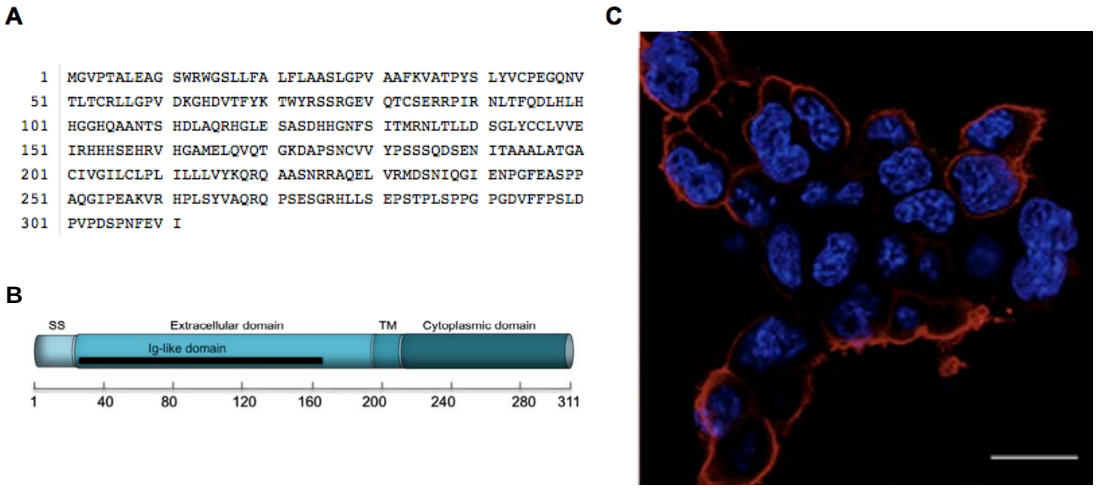


Figure 1 – Dies1 membrane protein. A. Amino acid sequence predict from a human *Dies1* gene. **B**. Dies1 protein predicted domains. Numbers indicates the aa position; SS, signal sequences; TM, transmembrane domain. Data provided in Ensemble database **C**. Immunostaining with a FLAG antibody demostrating the plasma membrane localization of Dies1 in mESC. Adapted from Aloia, el al. (1)

The mESCs transfected with shRNA targeting *Dies1* result in a decrease of specific neuron differentiated markers, even when cultured in a medium without the leukemia inhibitory factor (LIF, a factor required to sustain self-renewal of mESC). The suppression of *Dies1* expression affects not only neural, but also glial differentiation, and cells maintain the expression of markers of undifferentiated phenotype, such as *Oct4* and *Nanog*. *Dies1* Knock-down (KD) cells restore the proper differentiation ability after re-expression of *Dies1* gene (1).

The differentiation of mESC into cardiomyocytes is also impeded with *Dies1* silencing. Additionally, in both, neural and cardiac differentiation, *Dies1* mRNA expression levels increase when cells are cultured in differentiation promoting conditions (1).

An independent group also confirmed the function of Dies1 using a different differentiation program: adipogenesis. In this model the expression of *Dies1* transcript increases after one day of induction of adipogenic differentiation in 3T3-L1 preadipocytes; the mature adipocytes reach the maximum level of this transcript. Similarly to what was described by Aloia et al., the suppression of *Dies1* leads to partial inhibition of preadipocytes differentiation (5). Conversely, *Dies1* expression does not have a significant change during myogenesis (5).

The evidences collect by Aloia et al. and Ren et al., lead to the conclusion that *Dies1* is an important gene for cellular differentiation programs, in particular in cardiac, neural, and adipocyte differentiation (1, 5).

Following the protein structure and function Aloia et al. proved the function of Dies1 as a receptor protein. mESCs transfected with mutant *Dies1* (encoding only the N-terminal part

of the protein) had similar results to *Dies1* suppression results, suggesting that mutant *Dies1* function as a dominant negative form. However, Aloia et al. also showed that Dies1 extracellular domain by itself is not able to work as a soluble protein, suggesting that Dies1 has a signalling function inside the cell (1).

Overall, these results indicate that Dies1 functions as a surface receptor, possibly interfering with signalling pathways controlling mESC pluripotency (1).

1.1. The role of *Dies1* in the BMP4 signalling pathway

The LIF cytokine is necessary but not sufficient to maintain mESC undifferentiated in cell culture. Members of the transforming growth factor-β (TGF-β) family namely, bone morphogenetic proteins (BMP) and Nodal/Activin, have been reported to be necessary for the maintenance of mESC identity (6-8). In order to preserve the mESC phenotype, Dies1 possibly interacts with one of these pathways. Indeed, *Dies1* KD alters BMP4 signalling pathway without directly disturbing LIF and Nodal/Activin pathways (1). *Dies1* silencing in undifferentiated cells leads to a significant decrease in mRNA expression of BMP4 effectors, *Inhibitors of Differentiation* (*Ids*) *1, 2* and *3*, and also a reduction of phosphorylated Smad1/5/8 levels. Without directly affecting it, *Dies1* KD indirectly activates the Nodal/Activin signalling pathway, which reflects an increase expression of their downstream genes: *cripto*, *lefty1* and *lefty2* (1).

Bone morphogenetic proteins and Nodal/Activin are members of the TGF-β superfamily of cytokines, which are involved in a large variety of functions (9). BMP pathways are mainly involved in induction of cartilage and bone formation, cell differentiation, proliferation (10), apoptosis (11) and epithelial-mesenchymal transition (12, 13). Nodal/Activin signalling is implicated in development events and distinct studies have demonstrated that it is essential for embryonic stem cells maintenance (14). The TGF-β superfamily members initiate their cellular signalling pathways by binding the ligands to a heterotetrametric complex of type I and type II receptors (15). The constitutively active type II receptor kinase (TGF-βRII, BMPRII, ActRII and ActRIIB) phosphorylates the type I receptor, known as activin receptor like kinase (ALK-1 to ALK-7), that enables the recruitment and activation of the receptor-regulated Smads (R-Smads): Smad1, Smad2, Smad3, Smad5 and Smad8 (16). Activin and Nodal receptor type I specifically activates Smad2 and Smad3, whereas Smad1, Smad5 and Smad8 transduce signals in the BMP pathway. Activated R-Smads form a complex with the common mediator, Smad4, that translocate into the nucleus and interacts with various transcription factors to regulate the expression of target genes (16, 17). The ligand-dependent phosphorylation of R-Smads is inhibited by direct competition for receptor binding by the inhibitory Smads (Smad6 and Smad7)

(16). In parallel, the receptor complexes can also trigger diverse non-Smad pathways, such ERK, p38 and JUN N-terminal kinase (JNK) MAP kinase (MAPK), RHO-like GTPases and Phosphatidylinositol-4,5-bisphosphate 3-kinase (PI3K) (15, 17).

The BMP and Nodal/Activin pathways have a direct relation since they share the Smad4 cofactor (16, 17). The increased activity of the Nodal/Activin pathway, in *Dies1* KD cells, seems to be related with Smad4 availability. Indeed, even in the suppression of Nodal/Activin pathway activity, *Dies1* KD leads to a decrease of *Id1* and *Id3* mRNA levels, indicating that Dies1 acts directly on the BMP4-Smad1/5/8 pathway, and the Nodal/Activin pathway is only indirectly activated.

The interaction between Dies1 and the BMP4 pathway is reinforced by direct interaction of Dies1 with BMP4 cytokine, and by co-localization of Dies1 with the only BMP-type I receptor expressed in mESC: ALK3 (1, 18). All the evidences thus suggest that Dies1 plays a role as co-receptor in BMP4 pathway (1, 18). The suppression of Id1 protein, a BMP4 pathway target, is not sufficient to keep mESCs in their undifferentiated state. Therefore, in order to maintain this state by *Dies1* knockdown, Nodal/Activin activity is essential (1).

Dies1 suppression in mESC leads to a maintenance of stemness, and seems to depend on the equilibrium between Nodal/Activin and BMP4 signals (1).

In the adipogenesis model, *Dies1* KD leads to a partial impairment of differentiation. However, in contrast to what was reported for mESCs, the levels of BMP4-induced phospho-Smad1 did not decrease. Also during adipogenesis BMP4 transcript levels have an inverse relation with *Dies1* levels (5).

The enrolment of Dies1 in BMP4 signalling differs according to the differentiation model studied and needs to be further explored in order to clarify this association.

1.2. MiR-125 involved in Dies1 regulation

The small non-coding single-stranded ribonucleic acids (~22 nucleotides), microRNA (miRNA) family, play an important role in post-transcription regulation of gene expression in many biological processes, and aberrant or perturbation of their expression levels have an association with different disease, as an example of cancer (19-21). A key determinant for miRNA target recognition is based on base pairing of miRNA and the target mRNA. Usually, miRNA bind to mRNA in the 3′ untranslated regions (3′-UTRs), and many evidences suggest that miRNA do not function as naked RNA and they incorporate into a RNA-induced silencing complex also known as microRNA ribonucleoprotein complex (miRNP). The gene silencing can be obtained at 3 stages that include pretranslational,

cotranslational, and posttranslational steps and exert direct and indirect effects on translation machinery. MiRNA can mediate gene repression by deadenylation and decay of target mRNAs and repression of translation initiation that lead to degradation or storage of mRNA; or repress of protein expression by elongation block or photolytic cleavage of emerging polypeptides (21-23).

By crossing the nucleotide sequence of the 3'UTR *Dies1* mRNA with a list of miRNA previous found in ESCs (24), it was identified a possible binding site for miRNA-125a and 125b.

These two miRNAs belongs to the miR-125 family, which is highly conserved among mammals, is transcribed from 3 different clusters and composed by 3 homologs: hsa-miR-125a, hsa-miR-125b-1 and hsa-miR-125b-2 (25, 26). The members of this family play a role in many different cellular processes like cell differentiation, proliferation and apoptosis (25). MiR-125a was already described to play a role in ESC differentiation, by repress a considerable master regulator controller the pluripotency of ESC, *LIN28* (27), and by directly bind to the 3'UTR of Smad4, in a non conserved region between human and murine specimens, and promote human ESC entry into the neural lineage (28).

The study of miR-125 as a possible regulator of Dies1 expression was performed by Parisi, et al. The overexpression of both pre-miR-125a and 125b result in a decrease of Dies1 protein levels while the suppression of these 2 miRNAs resulted in the accumulation of Dies1 protein, without affecting the mRNA levels (18).

MiR-125a and miR-125b are differentially expressed in undifferentiated mESCs. Whereas the former is expressed in mESCs, the latter is almost undetectable and its expression increases during the first steps of differentiation (18, 29). MiR-125a was studied in mESC differentiation, and its overexpression recapitulates the effect of *Dies1* suppression, with the persistence of stemness characteristics and a decrease of *Id1* and *Id3* expression. (18). Moreover the co-transfection of pre-miR-125a with a vector containing *Dies1* lacking the 3'UTR, resulted in a normal differentiation with normal *Ids* levels. All this evidence indicates that, the effect of miR-125a overexpression in mESC pluripotency is mostly mediate by Dies1 (18).

The induction of BMP4 signalling pathway increases the levels of miR-125a and Dies1 protein, which in turn is markedly decreased after 24h of BMP4 treatment. This finding, together with the evidences that Smad1 interacts with miR-125a promoter region, suggests the existence of a negative feedback loop, where BMP4 controls Dies1 level through direct recruitment of Smad1 to the promoter of miR-125a gene (**figure 2**) (18).

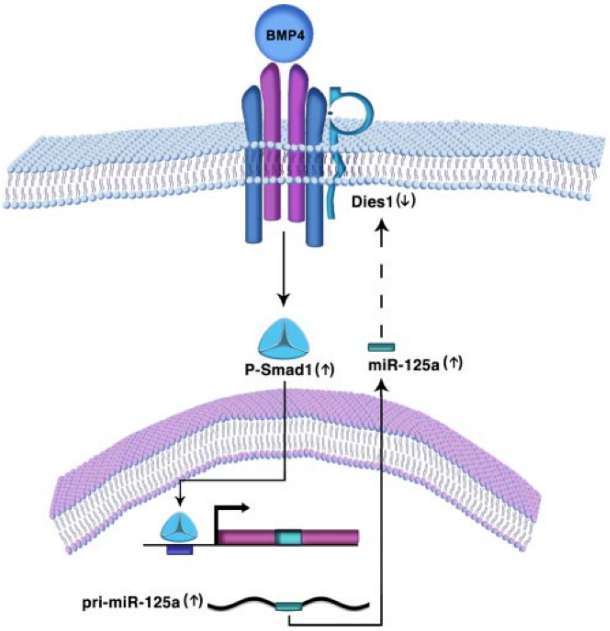


Figure 2 – Schematic representation of regulatory loop mediated by miR-125a. The treatment with BMP4 leads to an overexpression of miR-125a and down-regulates the Dies1 levels and decrease the BMP4 signal. Adapted from Parisi, el al. (18)

Through this regulatory loop mechanism BMP4 can modulate the sensitivity of the cells to itself. This can be important for mESC transition to epiblast stem cell (epiSC), that is block by BMP4 signalling (30). Indeed the overexpression of miR-125a that leads to Dies1 suppression and down-regulation of BMP4 signalling, resulting in ESC differentiation to epiSC (18). However the progression of the differentiation program appears to be slower and cells retain an epiSC phenotype while the levels of miR-125a remained elevated. This was concomitant to the overexpression of Nodal/Activin pathway that is known to be necessary to maintain the pluripotent state of mouse epiSCs (18).

Besides the low levels of miR-125b in ESCs, its expression increases during the first steps of differentiation. The study of this miRNA in ESC differentiation reveals that the overexpression of this miRNA blocks cells differentiation at epiblast stage and a large fraction of these cells maintain the pluripotent state (29). Also the Dies1 re-expression is able to fully rescue the block at the epiblast stage induced by miR-125b. Similar to miR-125a, miR-125b overexpression induced a significant decrease of BMP4 targets, and an increase of Nodal/Activin targets. However, contrary to miR-125a, miR-125b is not directly regulated neither by BMP4 neither by Nodal/Activin pathways, and the mechanism that regulate miR-125b remains unknown (29).

Until now, as far as we know, the regulation of Dies1 by overexpression of miR-125 is the only regulatory mechanism described in literature.

1.3. Dies1 in a non-differentiation context

Besides embryonic differentiation, others functions have been assigned to Dies1. A single report demonstrates that Dies1 protein serves as a substrate and a positive regulator of membrane-type1 matrix metalloproteases (MT1-MMP). The authors suggest that this protein also contributes to the invasive growth of HT1080 cells by up-regulation of the cell-surface MT1-MMP (3).

In 2011, Li Wang, demonstrated that the Dies1 protein, termed as VISTA in this report, exerts immunosuppressive activities on T cells. VISTA expression on antigen presenting cells directly suppresses T cell proliferation and cytokine production, and authors hypothesize that VISTA is an inhibitory ligand that down-regulates T cell-mediated immune responses (2). A recent study, in a mouse model, indicates that VISTA is highly expressed on tumour-infiltrating myeloid cells, and the monotherapy with VISTA monoclonal antibody (mAb) impairs the tumour growth in a bladder and melanoma tumour mouse models. VISTA blockage enhanced tumour-specific T-cell response, and increased the immune-stimulatory phenotype of dendritic cells (31). However another study described that an antibody to Dies1 protein, herein denoted PD-1H, is capable of modulating allogeneic T cell responses accompanied by inhibition of T cell accumulation and expansion in graft-versus-host disease in a mouse model (4). This study suggests, contrarily to previous, that PD-1H has a stimulatory role on T cell (4).

Further studies are, therefore, required in order to elucidate the function of this protein in both immunological and non-immunological context.

In summary, the relevance of *Dies1* in ESC differentiation has been demonstrated by several authors. Besides the involvement in ESC differentiation program, a preliminary study performed in our group has shown that *Dies1* may have a role in other differentiation/transdifferentiation programs, namely epithelial mesenchymal transition and the reversed process.

2. Epithelial Mesenchymal Transition and Mesenchymal Epithelial Transition

Epithelial and mesenchymal cells are one of the most primitive divergence cell phenotypes in early organisms (32). However, even in cells within a terminally differentiated state, these two phenotypes are not static and can be highly plastic and dynamic (32-34).

Epithelial to Mesenchymal Transition (EMT) is a complex process that enables the transdifferentiation (first cells de-differentiates and afterwards, cell differentiate into the new lineage) of epithelial cells to acquire an invasive and motile mesenchymal phenotype. Mesenchymal to Epithelial Transition (MET) is the reverse process (33, 35-38). EMT/MET represents a highly conserved biological process that normally occurs during development, wound healing, and regeneration, and has also been increasingly recognized in pathology, namely in fibrosis and cancer metastasis (33, 35, 36).

The EMT program allows the epithelial cells to loose the apical-basal polarity by dissolutions of cell-cell junctions, such as: tight junctions that is accompanied by decrease of claudin, occluding and zonula occludens (ZO) expression; loss the expression of adherent junction, epithelial cadherin (E-cadherin) that leading to nuclear accumulation of β-catenin and p120 catenin; disruption of desmosomes and; compromise the integrity of gap junctions. This process is also characterized by cytoskeletal architecture remodelling with formation of actin stress fibers and expression of mesenchymal cytoskeletal proteins such vimentin and reorganization of extracellular matrix (ECM) by up-regulating the expression of extracellular protein such fibronectin and collagens, proteases and others remodelling enzymes (39-41). Down-regulation of epithelial markers as E-cadherin, epithelial cytokeratins, occludins and claudins are accompanied by the increase expression of new mesenchymal markers, like N-cadherin, vimentin, fibronectin and α-smooth muscle actin (40, 41). All these alterations allow cells to acquire spindle shape morphology with cell protrusions and increased cell motility. Furthermore, cells develop resistance to senescence and apoptosis (32, 39, 42).

A high diversity of extracellular signals can trigger the EMT program, including soluble growth factors, such as TGF-β and epidermal growth factor (EGF), components of extracellular matrix, such as collagen and hyaluronic acid, and hypoxia (34, 41). In response to these signals, diverse signalling pathways active intracellular effector molecules, as example of small GTPase family (Ras, Rho and Rac) and members of the Src tyrosine-kinase family that are involved in disrupt of junction complexes and cytoskeletal organization. Several transcription factors, such as Snail1, Snail2, Zeb1, Zeb2, and Twist1 involved in either repression of epithelial or activation of mesenchymal

genes, are also activated during EMT (34, 39, 40).

2.1. E-cadherin expression alteration and "*cadherin switch*": the hallmarks of EMT/MET

Among many changes in gene expression, alterations on proteins functions in cell-cell and cell-matrix adhesion have a central role during EMT/MET. E-cadherin protein mediates epithelial cell-cell adhesion, and a decrease or loss of its expression constitute a well establish hallmark in EMT. E-cadherin down-regulation and/or dysfunction are found in all types of EMT in order to reinforce the destabilization of adherent's junctions (39, 43). In cancer the loss or abnormal expression of E-cadherin plays an important role as an invasion suppressor gene and are associated with tumour progression, metastasis and poorer prognosis in a variety of epithelial carcinomas, such as, gastric, breast, hepatocellular and colorectal carcinomas (44, 45).

E-cadherin is a member of the classical/type I cadherin family of cadherin superfamily, that count with 4 more members: placental (P)-, neural (N)- retinal (R)- and muscle (M)-cadherin (46). Classical cadherin family members have single-pass transmembrane glycoprotein that interact in a calcium dependent manner and establish a homophilic protein-protein interaction with cadherin molecules on the neighbouring cells (47-49). The intracellular domain of E-cadherin, interacts with some catenin proteins, β-, α-, γ-catenins to form a cytoplasmic cell-adhesion complex that links to the actin cytoskeleton through α-catenin and stabilize the cadherin-catenin complex (48, 50). Another catenin family member, p120-catenin, interacts with E-cadherin at its juxtamembrane domain and, not only stabilizes E-cadherin at cell membrane, but also prevents the protein from lysosome degradation. p120-catenin also interacts with the RhoA, Rac and Cdc42 (members of Rho GTPase), all key regulators of actin cytoskeleton organization (47, 49). Besides the adhesion function, E-cadherin is also involved in signalling, mostly by protein interaction with its cellular domain or by inhibition the ligand activation of receptors tyrosine kinase, such epidermal growth factor receptor (EGFR), human epidermal growth factor receptor 2 (HER2), the insulin-like growth factor 1 receptor (IGF-1R), and the hepatocyte growth factor receptor (HGFR). (47).

E-cadherin is an important hallmark of the EMT process, but how the loss of E-cadherin promote EMT and tumour invasion? (1) First, down-regulation of E-cadherin lead to a mechanical disrupt of adhesion junctions with loss of contacts among the neighbouring cells, leading to the detachment of malignant cells from the epithelial-cell layer (46, 48); (2) Second, cells become more responsive to tumour cell migration and tumour growth by several growth factors, such EGF, once cells lose the negative regulation provided by E-

cadherin (46, 48); (3) The disruption of E-cadherin cell-cell contact release proteins from the cell adhesion complex. These proteins have different functions depending on their cellular localization and with the loss of adhesion complex they become accessible to participate in alternative signalling pathways (43). One example is β-catenin, when it is released for cytoplasm, is stabilized by active Wnt signalling or by mutation in the β-catenin phosphorylation/degradation pathway, which allows its relocation to the nucleus, where it may activate the expression of Tcf/LEF complex. This complex modulates the expression of a large number of genes involved in cell proliferation, migration, invasion and morphogenesis, such c-myc, cyclin D1 and matrix metalloproteases (MMPs). Another example is the accumulation of p120-catenin in cytoplasm that is responsible for remodelling the actin filaments (33, 35, 43, 47); (4) As mentioned before, the suppression of epithelial markers is counterbalanced by increased expression of mesenchymal proteins. Specifically, E-cadherin down-regulation is balanced by increased expression of the mesenchymal cell-cell adhesion molecule, N-cadherin, that results in a *cadherin switch* with changes in cell adhesion (39, 51). N-cadherin is normally expressed in non-epithelial tissues, such nervous tissue, vascular endothelial cells, skeletal and cardiac muscle cells and is known as a *mesenchymal cadherin* (46, 47). E- and N-cadherin belong to the same cadherin family, and despite their structural and functional similarities, these 2 cadherins have different functions (46). In EMT the cadherin switch leads to an increase in the motility and invasion capability of cancer cells, and have been related with poor prognosis in multiple of cancers, such breast, prostate and pancreatic cancer (51). As a classical cadherin, N-cadherin has a homophilic binding pattern, and its expression lead epithelial cells to establish contacts with stromal and endothelial cells that normally express this cadherin. These contacts contribute to invasion and metastatic dissemination of cancer cells (46). N-cadherin also stimulates some signalling pathways that contribute to cell metastasis, by facility the binding of fibroblast growth factor 2 (FGF2) to its receptor and prevent the receptor internalization that sustain MAPK and PI3K/AKT signalling, leading to an increase of cell motility, invasion and segregation of extracellular proteases (48). N-cadherin might also activate platelet-derived growth factor (PDGF) receptor through association with a small protein called Na+/H+ exchanger regulatory factor (NHERF)-2, and induces actin reorganization and cell proliferation (47, 51).

The disruption of normal E-cadherin function and the switch to the expression of a mesenchymal cadherin involve many essentials changes that allows cell to undergo an EMT process and contribute for cell motility.

2.2. Role of epithelial mesenchymal transition in cancer

Contemplating the diversity of biological contexts in which EMT is involved, this process was classified into 3 different subtypes: the type 1 EMT is associated with embryonic implantation and development, such neural crest and fusion of the palate. Many cases of this subtype undergo MET to originate a secondary epithelia (42, 52); type 2 EMT is involved in wound healing, tissue-regeneration, inflammation, and organ fibrosis. The dedifferentiation and transdifferentiation of cells is generated to reconstruct tissues following trauma and inflammatory injury. In these case the process is induced in response to an inflammation, however when the inflammation insult is not attenuated the EMT process can contribute to organ destruction (42, 52, 53). The type 3 EMT occurs in carcinoma cells, however the involvement of EMT in cancer in not yet fully accepted across the scientific community (33, 54).

Since the report of Hanahan and Weinberg in 2000, metastases are recognized as one fundamental principle of the malignant transformation (55). This progress involves a series of sequential steps that culminate in establishment of tumour cells in distant organs. Initially cells must detach from primary tumour, became motile, degrade the basement membrane and ECM to invade the nearby tissue (invasion) and intravasate into circulatory system (intravasion). In circulation only a low number of cells can evade the immune system (systemic transport), extravasate through the capillary endothelium into the parenchyma of distant organs (extravasation) and proliferate to form a secondary tumour (colonization) (33, 56). Besides the existence of multiple models and patterns of invasion, mesenchymal single cell invasion has been highly accepted as a mechanism for cancer cell metastases, and many *in vitro* and *in vivo*, mainly mouse models demonstrate the importance of EMT in tumour progression (57, 58). The direct identification of EMT process in clinical specimens remains difficult, which leads to a scepticism to accept its pathological relevance. This difficulty is mainly due the existence of intermediate states of EMT, where a cell can acquire some mesenchymal characteristics, while maintaining some epithelial features; also because EMT is often a focal event, it may be missed in histological sections and under-represented in biopsy samples (56, 59, 60). Additionally, the fact that the EMT is a reversible program feeds the scepticism point of view (61).

The analysis of clinical samples reveals an increase expression of EMT markers in cancer cells on the invasive front of aggressive tumours. Also circulating cells from peripheral blood of breast cancer patients present an EMT phenotype and many tumour carcinomas, such colorectal cancer, the lost of E-cadherin expression are associated with undifferentiation cells (62-65). Beside these evidences of EMT process most of the

metastatic tumours present an epithelial phenotype, without any spindle morphology and with lack of EMT markers (62-65). This suggests that the EMT program is a dynamic process and the identification of cells with EMT characteristics may not be successfully achieved due the spatial and temporal heterogeneity of EMT in human cancer (56, 59). Jean Paul Thiery in 2002 (66) proposed a reversible EMT model, where primary tumour cells activate the EMT program, invade and disseminate, and after extravasion to a different organ, the cell reverts back to an epithelial phenotype by MET in order to facilitate colonization (56, 59). Supporting the sequential EMT/MET model, dynamic expression of E-cadherin has been documented in cancer progression, and multiples studies, using a mouse models, have demonstrated that tumour cells need to re-acquire an epithelial phenotype to form metastatic colonies (58, 67, 68). Tsai and colleagues demonstrated, using a mouse model study that a spatiotemporal regulation of EMT is essential for the success of metastasis to establish (58). Consistent with this work, Ocaña et al. demonstrated that the down-regulation of Prrx1 transcription factor, an EMT marker was associated to effective lung metastasis colonization (68).

All of these evidences are consistent with the role of the EMT/MET process in cancer. The transdifferentiation of cells from epithelial to mesenchymal cell type clearly illustrates the cell plasticity in EMT/MET program. Besides the cell transdifferentiation, some studies reported the association of this process with acquisition of stem cell-like characteristics (cell dedifferentiation), both by detecting expression of EMT features in $CD44^{+}/CD24^{-/low}$ cells purified from normal and malignant breast cancer tissue, and also by detecting increase of expression of cancer stem markers after induction of the EMT program, in different human cancer models (33, 69). Also the analyses of tumour tissue, such in claudin-low subtype of breast cancer, there was identified a correlation between gene expression signatures for both EMT and stem cells (70, 71).

3. Gastric cancer

Gastric cancer is the sixth most common cancer worldwide, and is responsible for about 9% of all cancer-related deaths. The incidence increases with the age reaching a pick in patients with 60-80 years and affecting more frequently the masculine sex (ratio: 2:1). Although the incidence of gastric cancer gradually decreased over the past 50 years, due mainly to reduction of chronic *Helicobacter pylori* (*H. Pylori)* infection, gastric cancer is still associated with poor prognosis, mainly in western countries (GLOBOCAN 2012) (72). The combination of late diagnosis and the lack of efficient treatment options in late stage disease lead to a low overall 5-years survival (about 25%). Survival increases to approximate 70% in Japan since this country presents a screening programme that allows the diagnosis at earlier stages of disease (73). The treatment of this cancer with curative proposes it's only possible by surgery and in the early stages; at advances stages regular chemotherapy is the standard treatment (73, 74).

The majority of gastric cancer (≈ 90%) appears in a sporadic setting, while the remaining 10% of the cases show familial clustering, and only 1-3% of these cases have a defined hereditary cause (73).

3.1. Gastric Cancer histological features and pathogenesis

Most of gastric cancer cases (≈ 90%) are gastric adenocarcinomas, an epithelial neoplasm, which displays high morphological heterogeneity (73, 75). This heterogeneity reflects the high diversity of different classification systems that has been proposed, such as Lauren, Ming, Carneiro, the World Health Organization (WHO) and the Japanese classification (76, 77).

Lauren classification is the most commonly used, this classification in based on tumour architectural and cytological features and individualises the tumours in two main types: intestinal and diffuse (78). All adenocarcinomas with predominant glandular epithelium, cells with intestinal columnar features and good cellular cohesion belong to the intestinal type (76, 77). By contrast, individual poor cohesive or small clusters of cells with little or no glands formation compose the diffuse type. These cells may contain mucus, which gives them a characteristic signet ring appearance (76, 77). In diffuse type usually exists an extensive newly formed stroma that difficult the identification of tumour cells by a routine coloration (76, 77). Broadly speaking, Lauren's diffuse type corresponds to an undifferentiated type, and the intestinal comprises the differentiated tumours. These two groups have different clinicopathological characteristics, distinct epidemiological settings and molecular pathogenesis. Intestinal type affects older patients, seems to have a

background of chronic gastritis and a high incidence of blood vessel invasion (76, 77). The diffuse type of gastric cancer affects patients earlier with a worse prognosis than intestinal type, the cells spread more commonly via the lymphatic system to pleura and peritoneum (76, 77).

Gastric cancer is thought to be the result of a combination of environmental factors such *H. Pylori* and Epstein-Barr vírus (EBV) infection, diet, and the accumulation of genetic alterations. The histological intestinal type, has been related with a multistep process in gastric carcinogenesis postulated by Correa et al., and based on epidemiological, pathological and clinical observation (79). The Correa's multi-stage cascade of gastric oncogenesis propose that, adenocarcinoma of intestinal type is the final stage of a serial of histological changes triggered by a long inflammatory process and followed by non-atrophic gastritis, multifocal atrophic gastritis, intestinal metaplasia and dysplasia (80). In contrast the development of gastric cancer diffuse type doesn't seems to follow this sequence of events and its pathogenesis remains unclear (76).

Additional to the intestinal and diffuse type, a third type was included latter in this histological classification in order to describe the carcinomas with uncommon or mixed histological features (**figure 3**) (76, 81).

Apart the morphological classifications others have been proposed based on mucin staining and tumour location and clinical course (82, 83).

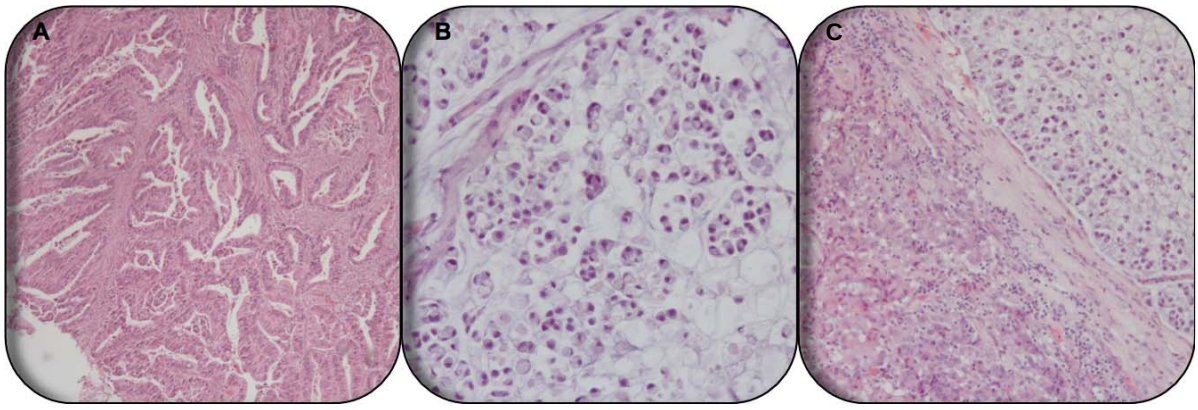


Figure 3 – Gastric adenocarcinoma histological subtypes. A. The intestinal subtype has a glandular architectural feature whereas **B**. a poor cohesive tumour cells are the main characteristic of the diffuse subtype. **C**. Additional to the major 2 subtypes the mixed subtype include an intestinal and diffuse histological features.

3.2. Gastric cancer under a molecular point of view

Together with the histological heterogenetic, also at biological level, gastric cancer is highly heterogeneous. Many efforts have been done to understand gastric cancer at the molecular level to complement traditional histopathological diagnosis, prognosis prediction and also to find new molecular alterations to be used in targeted therapy (84-86). Gastric cancer, as all cancers, is a chronic "proliferative" disease with multiple abnormalities that disturb a high variety of cellular functions, such cell cycle regulation and apoptosis (e.g. *cyclin E*, *cyclin D1*, *TP53*), DNA repair (e.g. *MLH1, MSH6, BAX*), chromatin remodeling (e.g. *ARID1A* and *MLL3*), cell adhesion (e.g. *CHD1*, *FAT4, RHOA*), invasion and angiogenesis (e.g. *VEGF*) and signalling pathways (e.g. Wnt, mTOR, TGF-β) (73, 77, 87, 88). All these abnormalities are due to changes in various oncogenes and tumour suppressor genes (TSG) and can be the result of multiple genetic alterations: point mutation, recombination, amplification, and/or deletion. Beside the genetic alterations, non-genetic changes such miRNA gene regulation and DNA methylation, have an important role in gastric cancer carcinogenesis (87, 89).

Gene amplification is one of the main alterations that drive carcinogenesis. The two growth factor receptors: EGFR (encoded by *EGFR*) and HER2 (encoded by *HER2*) are two members of the EGF receptor family altered in gastric cancer (87, 90, 91). *HER2* amplification has been reported in 6-32% of gastric cancer, mostly associated with intestinal type and, despite the controversy, seems to be associated with poor prognosis and tumour aggressiveness (92-94). Regardless the discrepant prognosis results and due the lack of treatment options for advanced stages of gastric cancer, U.S. Food and Drug Administration (FDA) and European Medicines Agency (EMA) approved transtuzumab (in combination with chemotherapy) as a treatment option for patients with HER2-positive metastatic gastric cancer (94). Similarly to *HER2* also *EGFR* amplification are present in this cancer and associated to lymph node metastasis, advanced tumour stage and poor survival (91). Additionally, *FGFR2* and *MET* (encode HGFR), which encodes receptors tyrosine kinases, and *KRAS* (encodes a membrane bound small GTPase protein) are also amplified in gastric cancer (95), Niantao Deng and co-workers, in a recent report using high-resolution single nucleotide polymorphism arrays, demonstrated that amplification of *KRAS*, *HER2*, *EGFR*, *FGFR2*, and *MET* present in 37% of gastric cancer patients are mutually exclusive to one another, and the possibility to apply a receptor tyrosine kinase/KRAS-directed therapy can constitute a good therapeutic approach (96, 97).

In addition to gene amplification, oncogenic mutations occurs in gastric cancer, examples of this is the activated mutations in *PI3K catalytic subunit alpha* and *KRAS*, that is a rare event in this cancer compared with amplification of this gene (73, 88, 89, 98).

Beside oncogene activation, a high number of TSG have been reported in gastric cancer pathogenesis, *TP53*, *RUNX3* and *CDH1* are three of these examples (99).

p53 (encoded by *TP53*), a nuclear protein involved in cell cycle, is the most studied TSG in cancer (100). The *TP53* is frequently inactivated in gastric cancer by missense mutations, which represent the higher mutations rate in this cancer, and by loss of heterozygosity (LOH). *TP53* mutations are not tumour specific and have been identified in pre-malignant lesions, also its alterations are not differently expressed in both histological types, and the prognostic impact of p53 alterations is still controversial (100).

RUNX3 (Runt-related transcription factor3, encoded by *RUNX3*) is a well establish tumour suppressor in gastric cancer that has an antiproliferative and apoptotic effects. Additionally, Voon, et al. and Peng Z and co-workers demonstrated, using animal models, an inversely association with loss or decrease in RUNX3 expression and increased of VEGF expression, elevated microvessel formation and EMT induction in gastric cancer cells (101-103). A reduction of RUNX3 transcription factor activity is due homozygous deletion, hypermethylation on promoter region, or protein mislocalization (103, 104).

The *CDH1* gene encoding homophilic cell-cell adhesion protein, E-cadherin, that has a pivotal function in maintain cell polarity and epithelial architecture (50). Alteration in its function has a role in tumour development and invasion and *CDH1* has been accepting as a TSG in gastric cancer (50, 105). About 90% of gastric cancer lose or have an aberrant E-cadherin expression, triggered by diverse mechanisms (50, 106, 107). Corso et al., studied the E-cadherin alterations in a gastric cancer cohort, and reported that somatic alterations occur in 30% of all gastric cancer cases (n= 246), 20% with promoter hypermethylation, and 10% with structural alterations (somatic mutations and LOH). However, more than 50% of patients with altered E-cadherin expression were negative for the alterations studied (108). Others alterations may have been present, such for example the deregulation of E-cadherin expression by miRNA such miR-101 and miR-200 family members and transcription factors repressors, aberrant glycosylation and proteolytic cleavage by MMP (50, 106, 107). In Corso et al. study, patients with *CDH1* structural alterations had a significant poorer survival rates than all others patients, and epigenetics alterations were more often present in diffuse histological type and associated with lymph node metastasis (108).

Regardless the *CDH1* specific alteration, deregulation of E-cadherin expression is often associated with cellular dedifferentiation, its expression reduction was associated with

poor overall survival, and decreases tumour cell sensitivity to conventional and targeted therapies (109-112).

E-cadherin can function as a biomarker in gastric cancer, and the *CDH1* inactivating mechanism may deliver information about the progression, invasion pattern, protein expression, and may even help predicting therapy response (91).

Apart to alteration in important cancer-associated gene, the loss of genomic stability is an important molecular step in gastric carcinogenesis, and creates a great environment for accumulation of other genetic and epigenetic alterations, either in tumour suppressor genes or in oncogenes (113). The loss of genomic stability has been identified in two phenotypes: (1) chromosomal instability, this is the most common genomic instability observed in solid carcinomas. These alterations are characterized by loss or gain of whole chromosome (aneuploidy) and/or factions of chromosomes (LOH, amplifications and translocations), and can lead to oncogene activation or TSG inactivation (113). Belien, el al., related that 80% of 221 tumour cases were classified as DNA aneuploid, and were associated with the intestinal type and presence of metastasis (114). (2) The second phenotype of genomic instability is microsatellite instability (MSI), defined as length change in repeating sequences of 1-6 base pairs of DNA (microsatellites) due to impaired of the DNA mismatch repair system. In many cases, MSI occurs due the hypermethylation of *MLH1* promoter. The frequency of MSI in gastric cancer is 15-20% of sporadic gastric cancer (depends on the series), and seems to be associated with older and female patients (113, 115, 116).

3.2.1. Molecular classification of Gastric Cancer

Many efforts have been done to achieve a molecular classification that can subdivide gastric cancer in different groups according to a biological profile.

Recently, Lei, et al., based on gene expression profiling of 248 gastric tumours, distributed gastric tumours into three subtypes, according to their biological characteristics and named them as: *mesenchymal*, *proliferative* and *metabolic* (85).

The *proliferative* type was characterized by a specific signature of genes related to the cell cycle and DNA replication with high activities for several oncogenic pathways: E2F, MYC, and RAS. These tumour present histological characteristics of intestinal type, at molecular level they are characterized by frequent *TP53* mutations and copy number alterations.

The second subtype of gastric cancer, *metabolic* subtype, showed a gastric phenotype, with high activity of pathways related to spasmolytic-polypeptide-expressing metaplasia (SPEM), an intermediate step in the Correa's multi-stage cascade of gastric oncogenesis

(79). Concerning treatment, this was the only sub-type for which patients benefited from 5-fluorouracil chemotherapy with better cancer-specific and disease-free survival (85).

Mesenchymal type, as the name suggests, had mesenchymal characteristic that resemble the EMT process: association with the Lauren's diffuse type, gene alterations are related with focal adhesion and extracellular matrix-receptor interaction and has some cancer stem cells (CSC) like proprieties. Additionally, cancer cells had high levels of *CDH2* and low levels of *CDH1*, similar to the classical *"cadherin switch"* in EMT (51). This subtype is also associated with TGF-β, vascular endothelial growth factor (VEGF), nuclear factor κ-light-chain-enhancer of activated B cells (NFκB), mammalian target of rapamycin (mTOR), and sonic hedgehod (SHH) pathways and showed higher proportion of aberrantly hypermethylated CpGs sites. Cell lines from mesenchymal subtype were more sensitive to phosphatidylinositor 3-kinase (PIK3)-AKT-mTOR pathway inhibitors, and these pathways have also been associated with EMT (39, 85).

3.3. Role of EMT in Gastric Cancer

The role of EMT in cancer is not yet completing accepted. Many *in vitro* and animal studies have supported that this process occur in cancer cells, however the relevance of EMT in human cancer is yet an open question in scientific community.

Besides the *in vitro* studies, clinical studies demonstrate that some cancer tissues display a dedifferentiation at invasive front regions, where cells lose their polarity and cell-cell contacts, leading to single cell detachment (117, 118). These alterations are associated with a *switch* in expression of some epithelial markers to mesenchymal markers in cancer cells, supporting an association with the EMT process (117, 118).

In several studies in gastric cancer, the EMT process seems to be related with tumour progression to metastasis. Iwatsuki, el al found that vimentin positive and E-cadherin negative cells invading the intratumoral vessels, and Yutaka et al. associated the expression of S100A4 protein (considered an mesenchymal marker) with lymph node metastasis and peritoneal dissemination. Also, node metastasis were associated with undifferentiated cells and reduced of E-cadherin expression (119, 120). These studies suggest that cells undergo a mesenchymal differentiation to invade and metastasize (119, 120). Others groups focus their studies on transcriptions factors associated with EMT and metastasis. Ru, et al., associated positive expression of Twist with depth of invasion and lymph node and distant metastasis (62). Also Snail1 and Zeb1 have been associated with metastasis and invasion in gastric cancer (121, 122). Using a tissue array methodology, Kim et al., performed an immunohistochemistry study with epithelial (E-cadherin, cytokeratin, β-catenin and γ-catenin) and mesenchymal markers (vimentin, N-cadherin,

MMP2, Snail1 and S100A4). After dividing the expression of these proteins in three groups according to number of proteins lost or gained in comparison to normal mucosa, a group with 4 or more protein changes was associated with poorly differentiated histology, advanced tumour invasion, lymph node metastasis and distant metastasis. Also, a multivariate analysis indicates that higher EMT-related protein changes were an independent prognostic indicator in cancer (123). With the same approach Ryu et al., indicated that the combination of epithelial and mesenchymal markers (Snail1, vimentin, E-cadherin and CD44) shows a significant association for determining prognosis for gastric cancer (124).

These two studies suggest that the combination of multiple markers expression more accurately predict patient outcomes compared with a single marker expression.

Besides the presence of mesenchymal proteins and the decrease of epithelial markers expression, some studies suggest the existence of cancer stem cells-like features associated with EMT markers, however few specific markers exists associated with these cells in gastric cancer, which turns their detection in tumour samples difficult (124, 125).

Additional studies based only in protein expression, demonstrate the presence of EMT in gastric cancer, an example is the recent study performed by Lei, et al. (described above) (85).

Many of these studies demonstrate that gastric cancer is a highly heterogeneous tumour type, not only at the cellular but also at the molecular level. As many epithelial neoplasms, this heterogeneity is frequently accompanied by the loss of cell differentiation and also some studies show that gastric cancer cells can additionally suffer a dedifferentiation and transdifferentiation program (EMT) to enhance their ability to metastasise.

The continuous search for new biomarkers to better characterize this cancer is essential to understand the cancer cell biology, which may help on the development of new diagnosis and treatment approaches, and also be useful for prediction of prognosis and treatment response.

The *Dies1* gene, recently described in a differentiation and transdifferentiation context can also be involved in cancer, since many cancer cells lose their differentiation, and can suffer a transdifferentiation through EMT process. The study of the involvement of this gene in a cancer model, gastric cancer, constitutes the major aim of this thesis.

CHAPTER 2

RATIONAL AND AIMS

Dies1 has been associated with differentiation contexts, not only in ESC differentiation, but also in EMT/MET, a process characterized by transdifferentiation of epithelial cells. In mESC, *Dies1* expression increase during neuronal and adipogenesis differentiation programs and its suppression maintains cell in an undifferentiation state. During the EMT/MET program, *Dies1* seems to follow a classical epithelial behaviour, with the decrease of its expression, when cells acquire a mesenchymal phenotype, and an increased expression at same time that the cell re-acquires the epithelial characteristics. Similar to what occurs in ESC differentiation and in an *in vitro* EMT/MET process, **we hypothesized that the loss of epithelial cancer cell differentiation may be accompanied by alterations of *Dies1* expression.**

Gastric carcinoma is one of the examples where cancer cells lose their epithelial features by disrupting the normal E-cadherin expression, and sometimes by gaining mesenchymal-like features. Besides these characteristics, preliminary data suggests that *Dies1* gene can be differently expressed in this cancer model.

The main aim of this study was **to dissect the role of *Dies1* in gastric cancer and its possible expression regulatory mechanisms.** To achieve this goal, 2 specific objectives were outlined:

1. Characterize the *Dies1* expression in gastric cancer cell lines and tumour samples, and explore the possible expression regulatory mechanisms of this gene;

2. Analyse the expression relationship of *Dies1* with an epithelial (E-cadherin) and a mesenchymal (N-cadherin) marker.

CHAPTER 3

MATERIALS AND METHODS

1. Cell lines and cell culture conditions

Human gastric cancer cell lines, AGS, KATO III, MKN28 and MKN45 were provided from American Type Culture Collection (ATCC, Manassas, VA, USA). Two non-commercial cell lines, GP202 and IPA220, were established and characterized at IPATIMUP (126). The cell lines were maintained in RPMI 1640 Glutamax I supplemented (Gibco, Invitrogen, Oregon, USA) with 10% (v/v) fetal bovine serum (Biowest, Nuaillé, France) and 1% (v/v) penicillin-streptomycin (10,000 U/mL, Invitrogen, Oregon, USA) medium and cultured in a humidified 5% CO_2 incubator at 37ºC.

2. Gastric cancer and normal paired samples

Tissue specimens of gastric cancer (n= 30) and matched non-cancer samples, included in this study, were obtained from the Tumour Bank at Hospital S. João under an institutionally approved protocol and with patient informed consents. All tumour samples were classified as adenocarcinomas and subdivided, according to the Lauren classification, into intestinal (n= 16) and diffuse (n= 14) types. Samples fulfilled a gross examination and representative samples of tumour and non-neoplastic tissue was collected and immediately snap frozen in liquid nitrogen (127). A “mirror/twin” sample of the frozen tissue was paraffin embedded and heamatoxylin and eosin (H&E) staining was performed for morphological characterization. The gross examination and the morphological characterization of all tumour samples were performed by the pathologists at Hospital S. João.

3. RNA extraction

3.1. Cell lines

RNA from cell lines samples was extracted using the *mirVana miRNA Isolation Kit* (AM1560, Ambion**,** Austin, TX) following the total RNA isolation procedure (including small RNAs), which combines 2 RNA extraction methods, namely, organic and solid-phase extraction. Initially the samples were disrupted in a denaturing lysis buffer, after which an acid-phenol:chloroform extraction was performed. Total RNA purification was performed using a filter cartridge containing a glass-fiber filter on which the RNA is immobilized. After this procedure the filter was washed and the RNA was eluted with 50µl of UltraPure

DNAse/RNAse-Free Distilled Water (Gibco, Invitrogen, Oregon, USA). The RNA quantity was assessed by spectrometry using a NanoDrop spectrophotometer (ND-1000 UV-Vis spectrophotometer, Thermo Fisher Scientific).

3.2. Tissue specimens

A volume of approximately 25-30 mm^3 was sectioned from each frozen tissue sample and cut in small pieces. Subsequently, the tissue was disrupted and homogenized in 700µl a Qiazol lysis reagent (QIAGEN, Hilden, Germany) using a Tissueruptor, a handheld rotor-stator homogenizer (QIAGEN, Hilden, Germany). After 5 minutes of room temperature incubation, 140µl of chloroform was added to the sample. After being incubated for another 5 minutes the sample was centrifuged for 15 minutes at 12000x g at 4ºC in order separate the homogenate into an aqueous and organic phases. The aqueous phase was subjected to automatic total RNA extraction in a robotic workstation, QIAcube equipment (QIAGEN, Hilden, Germany), where all the purification procedure was completed using a spin-column kit (miRNeasy Mini Kit, QIAGEN, Hilden, Germany). The total RNA was eluted in 50µl of UltraPure DNAse/RNAse-Free Distilled Water (Gibco, Invitrogen, Oregon, USA), quantified by spectrometry (NanoDrop spectrophotometer), and its fragment size distribution was analyzed using the Aligent 2100 Bioanalyzer.

4. RT-PCR and quantification of *CDH1*, *Dies1* (*C10orf54*) and *CDH2* mRNA expression

A first-strand complementary DNA (cDNA) was obtained from 1µg of total RNA from gastric cancer cell lines, commercial total RNA from normal stomach acquired from *Stratagene* (540037-41, Agilent Technologies, Inc., TX, USA), gastric tumours, and normal gastric mucosa using random hexamer primers (Invitrogen, Oregon, USA). The RNA sample and random primers were incubated for 10 minutes at 70ºC and then placed for 2 minutes at 4ºC. The first-strand reaction was catalysed by SuperScript Reverse Transcriptase II (Invitrogen, Oregon, USA) for 60 minutes at 37ºC.

Quantitative real-time PCR (qRT-PCR) was carried out on an ABI Prism 7000 Sequence Detection System (Applied Biosystems, California, USA) using the oligonucleotide probe approach. Gene expression detection was performed in triplicate for *CDH1*, *C10orf54* (*Dies1*), and *CDH2* genes, as well as for the endogenous controls *GAPDH* or *18s* RNA, using as probes sets: HS.PT.49a.3324071, Hs.PT.58.875102, Hs.PT.58.45367437, Hs.PT.51.1940505 (Integrated DNA technologies, Iowa, USA), Hs99999901_s1 (Applied Biosystems, California, USA), respectively. PCR reaction of the cell lines was performed

using 50ng of cDNA and the TaqMan Universal PCR Master Mix (Applied Biosystems, California, USA) in a PCR program with a holding stage of 20 seconds at 50ºC and 10 minutes at 95ºC, and 40 cycles of 15 seconds at 95ºC and 1 minute at 60ºC. For frozen samples a fast-cycling probe-based real-time PCR, Kappa probe fast qPCR kit (KapaBiosystems, MA, USA) with the Rox low reference dye was used, with the following PCR program: 95ºC for 20 seconds to enzyme activation and 40 cycles of 95ºC for 3 seconds and 30 seconds at 60ºC. Data were analyzed with the comparative C_t ($2^{-\Delta\Delta Ct}$) method.

5. RT-PCR and miR-125a expression quantification

The quantification of mature miR-125a-5p expression was carried out using the TaqMan microRNA assay in a two-step qRT-PCR. The reverse transcription was performed with 10ng of total RNA from gastric cancer cell lines and commercial RNA from normal stomach (540037-41, Stratagene, Agilent Technologies, Inc., TX, USA), a stem-loop RT specific primer, hsa-miR-125a-5p (RT:002198, Applied Biosystems, California, USA) for miR-125a and, NR_002745 (NCBI Accession) (RT:001006, Applied Biosystems, California, USA) for RNU48 and the TaqMan MicroRNA reagents (Applied Biosystems, California, USA) according to manufacturer instructions. The first-strand complementary DNA conversion reaction was performed in a thermal cycler using the following program: 20 minutes at 16ºC, 30 minutes at 42ºC and 5 minutes at 85ºC. In the PCR step, approximately 1.7ng of RT-product was amplified with the TaqMan Universal PCR Master Mix and the specific probes hsa-miR-125a-5p (TM:002198, Applied Biosystems, California, USA) for target miRNA and NR_002745 (NCBI Accession) (TM:001006, Applied Biosystems, California, USA) for endogenous control (RNU48). Real Time PCR reactions were performed in triplicate with negative control for each probe using the following PCR program: 10 minutes at 95ºC, and 40 cycles of 15 seconds at 95ºC and 1 minute at 60ºC. Data analysis was carried out with the comparative C_t ($2^{-\Delta\Delta Ct}$) method.

6. DNA extraction

6.1. Cell lines

DNA extraction from cell lines was performed using and tracking the Invisob Spin Tissue Mini Kit protocol (STRATEC Molecular, Berlin, Germany). Cells were detached with trypsin, and the pellet incubated with proteinase K overnight at 52ºC under constant shaking (80rpm). After the incubation step, samples were centrifuged at maximum speed and the cleared supernatant was carefully transferred to a new 1.5ml tube. DNA

purification was accomplished using a silica-based method, and the DNA was eluted in 50µl of UltraPure DNAse/RNAse-Free Distilled Water (Gibco, Invitrogen, Oregon, USA). DNA quantification was performed by quantitative spectrophotometric assay using a NanoDrop spectrophotometer. A purified DNA sample was considered with an absorbance quotient value of $1.8< OD_{260}/OD_{280} <2.0$.

6.2. Frozen samples

Similarly to RNA extraction, DNA extraction from frozen tissues was fulfilled in a robotic workstation, QIAcube (QIAGEN, Hilden, Germany), using a QIAamp DNA Mini Kit (QIAGEN, Hilden, Germany). Approximately 25-35mg of tissue was cut in small pieces, transferred to a cold 2ml tube with ATL buffer and homogenized using a Tissueruptor (QIAGEN, Hilden, Germany). For protein digestion the samples were incubated overnight with proteinase K at 55ºC on constant shaking at 240rpm. After AL buffer addition, the samples were transferred to the QIAcube equipment in order to complete the DNA extraction protocol. DNA concentration was measure by spectrometry (NanoDrop spectrophotometer) and DNA quality was verified by a 1% agarose gel electrophoresis.

7. Bisulfite treatment

In order to predict the existence of CpG islands in *Dies1* promoter region a bioinformatical analysis was completed using the following criteria: a) genomic length with ≥500 bp; b) a percentage of GC content ≥55%; and c) CpG dinucleotides observed/expected ratio ≥0,65. This analysis was performed using the Ensemble database and the web tool "*CpG Island Searcher*", which predicted a single CpG island at gene promoter region. The 50 CpG sites analyzed where located at chr10: 71773310-71773801.

The method used to analyze the promoter methylation status of the *Dies1* gene was sodium bisulfite conversion. Treatment with sodium bisulfite leads to a deamination of unmethylated cytosines to uracils, while leaving methylated cytosines unchanged since the deamination rate of 5-methylcytosine (5mC) to thymine is much slower (128). Accepting the different rate of deamination, it is further assumed that cytosines remaining after the treatment were derived from 5-methylcytosine. Finally, the treatment products are amplified and sequenced for methylation status determination (128, 129).

Approximately 500ng of cell lines and tumour DNA were used for sodium bisulfite treatment with the EpiTeck Bisulfite kit (QIAGEN, Hilden, Germany). The bisulfite conversion was performed by mixing the DNA solution, bisulfite mix, DNA protector buffer, and DNase/RNase-free water in a thermal cycler using the following protocol: 5 minutes

at 95ºC, 25 minutes at 60ºC, 5 minutes at 95ºC, 85 minutes at 60ºC, 5 minutes at 95ºC and 125 minutes at 60ºC. The desulfonation and DNA purification was completed in an EpiTeck spin column membrane. DNA was further eluted in 20µl of UltraPure DNAse/RNAse-Free Distilled Water (Gibco, Invitrogen, Oregon, USA). Bisulfite treatment DNA was amplified using primers specifically designed for bisulfite treated DNA sequences without CpG sites (*Sigma-Aldrich*, Missouri, USA) The PCR reaction forward and reverse primers are listed in **table 1**. After amplification, the PCR products were purified using Exonuclease I (Ecoli, 20 U/µl, Thermo scientific, Cheshire, UK) and FastAP Thermosensitive Alkaline Phosphatase (1 U/µL, Thermo scientific, Cheshire, UK) enzymes during 15 minutes at 37ºC and 15 minutes at 85ºC. For methylation status determination, the purified PCR products were sequenced using the primers listed in **table 2** and the following program: 2 minutes at 96ºC, 30 cycles of 30 seconds at 96ºC, 30 seconds at 54ºC and 3 minutes at 60ºC; and 10 minutes at 60ºC.

In the methylation status analyses all unmethylated cytosines (C) were converted to a thymidine (T), and the presence of a C-peak indicated the presence of 5-methylcytocine in the genome. A double peak (C andT) indicated that the CpG site was partially methylated.

Table 1 – Sequence of the primers used for PCR amplification of bisulfite treated DNA.

Amplicon ID	Forward primer	Reverse primer	PCR Product Length
1	5'-GTTAGAGGTAGATTTATTTTTTAGGTTG-3'	5'-CTATCTTCTCCCAACTTTTTCC-3'	492 bp
2	5'-GGAGAGGTAGTTTTTTTATA-3'	5'-CTATCTTCTCCCAACTTTTTCC-3'	316 bp

bp, base pairs.

Table 2 – Sequence of the primers used for bisulfite treated DNA sequencing.

Forward primer
5'-GTTAGAGGTAGATTTATTTTTTAGGTTG-3'
5'-GGAGAGGTAGTTTTTTTATA-3'
5'-GGTATTAGAAGTTTTTTTG-3'
Reverse primer
5'-CTATCTTCTCCCAACTTTTTCC-3'
5'-TATAAAAAAACTACCTCTCC-3'
5'-CAAAAAAACTTCTAATACC-3'

8. *Dies1* mutation screening

To accomplish the sequencing of the entire coding sequence of *Dies1* gene and the predicted target site of miR-125a nine pairs of primers were designed. The designed of primers were performed using: (1) Ensemble database to search for the desired sequence

and to confirm the specificity of the primer sequence for our target sequence, using for that the "*Blast*" web tool; (2) and the Primer3web software (version 4.0.0) for achieve the primers sequence.

After primer conditions optimization, *Dies1* mutations screening was attained using 2 independent multiplex polymerase chain reactions (PCR), while exons 1, 7 and the latter sequence of exon 2 were independently amplified. Multiplex mix 1 contained 3 primers sets in order to amplify exons 3, 6 and the predicted target site of miR-125a and the multiplex mix 2 contained primers sets for the initiation sequence of exons 2, 4, and 5 (primers sequences are represented in **table 3**). Approximately 120ng of DNA was used for the amplification reaction, whereas a double of DNA quantity was required for exon1 amplification, using for PCR reaction a multiplex mix with a 0.2µM of primers and Q-solution reagent (QIAGEN, Hilden, Germany) (PCR programs are shown in **table 4**). The status of amplification and contamination of PCR products was evaluation by a 2% of agarose gel electrophoresis, and the products were purified by enzymatic methods using Exonuclease I (Ecoli, 20u/µl, Thermo scientific, Cheshire, UK) and FastAP Thermosensitive Alkaline Phosphatase (1 U/µL, Thermo scientific, Cheshire, UK) enzymes or by gel band extraction and purification using illustra GFX PCR DNA and a Gel band purification kit (GE Healthcare, Buckinghamshire, UK). PCR products purification was sequenced using the primers represented in **table 3**, and PCR program are described in bisulfite treatment section.

Table 3 – Sequence of the primers used in *Dies1* mutations screening for polymerase chain reaction.

Reaction	Forward primer	Reverse primer	Sequence amplified	PCR Product Length
Multiplex mix 1	5'-CTAGCGTGAGAACCTGGGG-3'	5'-TGAGGCCAGAGTTCCAAACA-3'	Exon3	250 bp
	5'-GGGATGGGGATGTAGGTGAG-3'	5'-CTCTTCAGGGAGGGCAGG-3'	Exon 6	400 bp
	5'-ATGAATGAGGCCTTCCCCAG-3'	5'-CCATGGGGTGAAGAGAAGCA-3'	Predict target site of miR-125a	243 bp
Multiplex mix 2	5'-GGCAGTCTTAGGGAGGTCAG-3'	5'-TGGTGATGGAGAAGTTGCCA-3'	Initial seq. of exon 2	456 bp
	5'-CTTCCTGGCCTTCCCTCTC-3'	5'-AAGGAATTGTGCCTGCATCG-3'	Exon 4	306 bp
	5'-AGGGGCAGGGATGTTGTATG-3'	5'-GCCCCTACTGCACAGAAGTA-3'	Exon 5	175 bp
Reaction 3	5'-CGGTAGAACGGGCTTTAAACTG-3'	5'-GAGTGGGGTGCACGGTCA-3'	Exon 1	502 bp
Reaction 4	5'-CTCACGTTCCAGGACCTTCA-3'	5'-GAGTGCCAGTGTTACCCATG-3'	Final seq. of exon 2	360 bp
Reaction 5	5'-GGGATGGGGATGTAGGTGAG-3'	5'-CTCACAGAGCCAGCCCTG-3'	Exon 6 and 7	792 bp

bp, base pairs; seq, sequence

Table 4 – PCR amplification program used in *Dies1* mutations *screening*.

Step	Temperature	Time	Number of cycles
Initial denaturation and polymerase activation	95ºC	15 minutes	1
Denaturation	94ºC	30 seconds	2
Annealing	64ºC / 60ºC (reaction3)	90 seconds	
Extension	72ºC	90 seconds	
Denaturation	94ºC	30 seconds	3
Annealing	62ºC / 58ºC (reaction3)	90 seconds	
Extension	72ºC	90 seconds	
Denaturation	94ºC	30 seconds	30
Annealing	60ºC / 56ºC (reaction3)	90 seconds	
Extension	72ºC	90 seconds	
Final extension	95ºC	15 minutes	1

9. Immunohistochemistry: anti-Dies1 antibody optimization

The antibody optimization was performed in human and mouse tissue species due the availability of the tissue material and the necessity to study this protein in the mouse tissues.

9.1. Tissue microarray construction

For construction of a test tissue microarray (TMA) for antibody optimization a diversity of tissues were collected from a healthy CBA/N strain mice. The tissues collected were immediately immerse in formalin solution, neutral buffered, 10% (approx. 4% formaldehyde) and after approx. 55 hours, samples were processed in an automatic tissue processor with new reagents to ensure the quality of antigens epitopes, and using the followed program: 1 hour in 70% ethanol, 1 hour in 96% ethanol, 3 baths of 1 hours in absolute ethanol and 3 baths of 1 hour in heat paraffin wax. The tissues were then embedding in an orientation that allowing see all the organ structures, and 2/3µm thick section was performed. Tissue sections were then stained with a routine H&E coloration and the interested structures were selected and marked on paraffin blocks (**figure 4A** and **B**). After the selection of the interesting structures a TMA gird was generated using the Excel datasheet that contains the tissue coordinates. Some features were taking in account in the gird construction: the gird pattern was not completely symmetrical and the liver, a characteristic tissue, was included alone in one extreme of the TMA in order to

facility its orientation (**figure 4C**). In this work was used the *Arraymold Manual Tissue Microarrayer kit* (IHC World, LLC, MD, USA) for TMA construction. A receptor block was construction using the reusable rubber mold provided by the kit, the receptor block had 35 cores holes, each one with 3mm of diameter. Using the disposable 3 mm sterile dermal biopsy punch, a core of tissue was extracted from selected area of donor block and insert in the core hole of receptor block following the X/Y coordinates of the gird pattern previously designed (**figure 4D and E**). Cores holes with no tissue designated were filled with a core of paraffin wax. Once all cores holes were completed, the recipient block was slightly melted at 37ºC overnight in order to bind the cores into the block, and avoid their loss in the section procedure. During this procedure a glass slide was adherent to the cores to obtain a plane surface and perform a uniform section. After a period of room temperature resting the slide was slightly removed and sequential 2-3µm sections were performed and adherent to a coated glass slide (Superfrost Plus, Gerhard Menzel, Braunschweig, Germany). An H&E staining was done to the first section and every after 10 sections made, to address if the selected tissue areas were still represented in the TMA block.

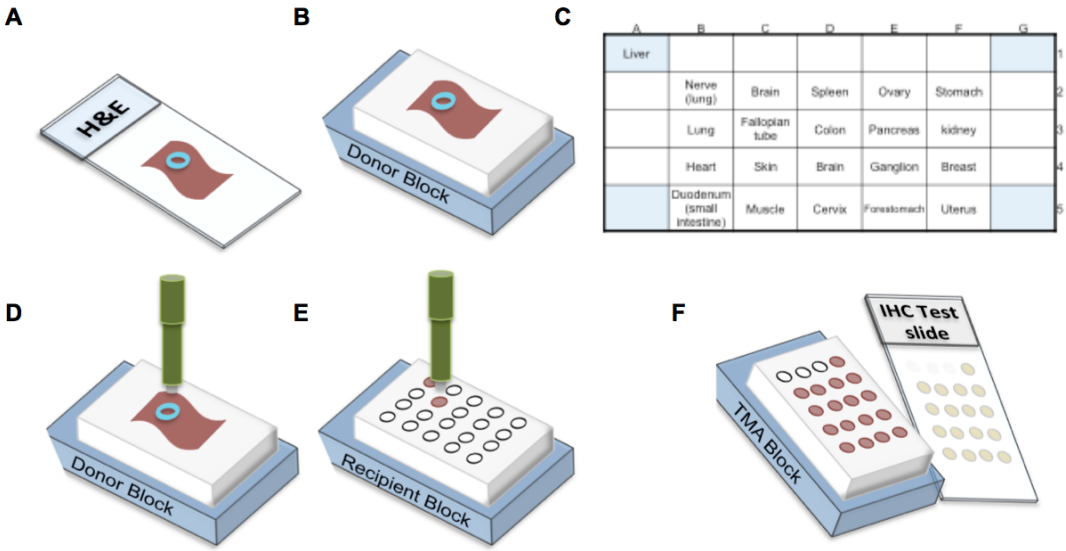


Figure 4 – Steps for tissue microarray construction. Selection of interesting structures on **A.** H&E slide and **B.** paraffin donor block. **C.** TMA gird generated on Excel database. **D.** Extraction of a tissue core from the selected area on the donor block. **E.** Core of tissue insert in the recipient block. The steps **D** and **E** were repeated until complete the predefined TMA plan. **F.** Final TMA block and test slide.

9.2. Immunohistochemistry technique

Immunohistochemistry (IHC) optimization was performed in TMA sections for mouse tissues and for human tissue optimizations this was fulfilled mainly in normal gastric tissue, however others tissues were used, such normal spleen, testicle, thyroid, breast and placenta tissue. Sections (3µm thick) from paraffin-embedded tissues were deparaffinized in xylene and hydrated through a decreasing graded ethanol series. Antigen retrieval was performed using 2 different methods: proteolytic and heat induced epitope retrieval. In the first method a commercial pepsin solution (DIGEST-AL 3, Invitrogen, Oregon, USA) was used for 10 minutes at 37ºC; in the second 2 different heat approaches were tested: microwave and IHC-Tek Epitope Retrieval Steamer Set with 2 different solutions, namely citrate (pH6) or ethylenediaminetetraacetic (EDTA, pH8) buffer (Abcam, Cambridge, UK). Slides were allowed to cold down to room temperature, rinsed in Tris-buffer saline containing 0.02% Tween 20 (TBS-T) (AMRESCO, OH, USA) at pH 7.6, and the endogenous peroxidase was blocked with 3% hydrogen peroxide in methanol for 15 minutes. Subsequently, the slides were washed in TBS-T, treated with V-block solution (TA-125-UB, Thermo scientific, Cheshire, UK) to block nonspecific protein binding and incubated with anti-Dies1 antibody (MAB7126 clone 730802 or MAB71261, clone 730804, R&D Systems) (the specific conditions of each optimization test are described in **table 5**). After being rinsed in TBS-T, the antibody-antigen complex was detected with a horseradish peroxidase (HRP) polymer system (Dako REAL EnVision HRP rabbit/mouse, Dako, Glostrup, Denmark) for 30 minutes at room temperature, and the antigen localization was visualized with 3, 3'-diaminobenzidine (DAB) solution. Finally, the sections were counterstained with Gill's haematoxylin No.2 (Bio-optica, Milano, Italy), dehydrated, and mounted using a Richard-Allan Scientific Mounting Medium (Thermo Fisher Scientific, MA, USA).

The **table 5** summarizes all the tests performed in order to optimize Dies1 immunostaining.

Table 5 – Immunostaining conditions tested for anti-Dies1 antibody optimization.

Optimization test	Antigen Retrieval conditions	Antibody	Antibody dilution	Antibody conditions
1	IHC-Tek™ Epitope Retrieval Steamer Set, 40 min, Citrate Buffer	R&D Systems, clone 730802	1:100	Overnight, 4ºC
2	IHC-Tek™ Epitope Retrieval Steamer Set, 30 min, Citrate Buffer		1:100	
3	IHC-Tek™ Epitope Retrieval Steamer Set, 40 min, EDTA Buffer		1:100	
4	Microwave, 20 min, Citrate Buffer		1:100	
5	Proteolytic digestion, 10 min, 37ºC		1:100	
6	-		1:100	
7	IHC-Tek™ Epitope Retrieval Steamer Set, 40 min, Citrate Buffer	R&D Systems, clone 730802	1:50	Overnight, 4ºC
8	IHC-Tek™ Epitope Retrieval Steamer Set, 40 min, Citrate Buffer		1:75	
9	IHC-Tek™ Epitope Retrieval Steamer Set, 40 min, Citrate Buffer		1:150	
10	IHC-Tek™ Epitope Retrieval Steamer Set, 40 min, Citrate Buffer	R&D Systems, clone 730802	1:100	1hour, Room Temperature
11	IHC-Tek™ Epitope Retrieval Steamer Set, 40 min, Citrate Buffer		1:100	30 minutes, 37ºC
12	IHC-Tek™ Epitope Retrieval Steamer Set, 40 min, Citrate Buffer	R&D Systems, clone 730804	1:50	Overnight, 4ºC
13	IHC-Tek™ Epitope Retrieval Steamer Set, 40 min, EDTA Buffer		1:50	
14	Proteolytic digestion, 10 min, 37ºC		1:50	
15	IHC-Tek™ Epitope Retrieval Steamer Set, 40 min, Citrate Buffer	R&D Systems, clone 730804	1:50	3hours, Room Temperature
16	IHC-Tek™ Epitope Retrieval Steamer Set, 40 min, Citrate Buffer	R&D Systems, clone 730804	1:30	1hour, Room Temperature

10. E-cadherin and N-cadherin Immunohistochemistry

Automatic immunostaining was performed for E-cadherin and N-cadherin proteins using the BenchMark ULTRA, IHC/ISH slide staining system (Ventana Medical Systems, Inc, Roche, AZ, USA), after manual optimization of antibodies conditions.

Formalin-fixed, paraffin-embedded tissue sections (3µm thick) were deparaffinized for 4 minutes at 72ºC, using EZ-Prep solution (Roche, USA). Tissue pre-treatment was conducted with a heat induced epitope retrieval (Roche, USA), at 95ºC for 8 minutes, and the endogenous peroxidase was inactivated with UV Inhibitor solution for 4 minutes at 36ºC. After several washes with the Tris based buffer solution (reaction buffer, pH7.6,

Roche, USA), the tissue section was incubated with primary antibody, anti-E-cadherin (1:50 dilution; clone 4A2C7; Zymed, South San Francisco, CA) for 28 minutes at 37ºC or anti-N-cadherin (1:650 dilution; 610920; BD Transduction Laboratories, New Jersey, USA) for 32 minutes at 37ºC. An indirect, biotin-free system, Ultraview Universal DAB Detection Kit (Roche, USA) was used to detect the antigen-antibody complex. For this purpose the samples were incubated with Ultraview universal HRP multimer (Roche, USA) for 8 minutes at 36ºC, and with Ultraview universal DAB chromogen for 8 minutes. The contrast step was performed automatically using the Hematoxylin reagent (Roche, USA) for 8 minutes, and the dehydrated, and mounted steps were manually executed.

11. Immunohistochemistry evaluation

IHC results for E-cadherin and N-cadherin were evaluated by a pathologist and scored according to the percentage of positive cells, cellular localization of protein, and intensity of protein expression (relative to normal expression). E-cadherin expression in normal glands and N-cadherin expression in ganglion cells of Meissner's plexus or Auerbach's plexus served as positive control. An external control, testis sample, was also including in N-cadherin staining.

The staining extension pattern was measured using the following classification: (0), 0-25% of positive tumor cells; (1+), 26-50% of positive tumor cells; (2+) 51-75% of positive tumor cells and (3+) > 76% of positive tumor cells. For intensity evaluation purposes these markers were considered negatives when 0 was scored, and positive staining was scored as 1+, 2+, or 3+. The E-cadherin protein cellular localization was classified as normal (membrane expression) or aberrant expression (cytoplasmic localization, membrane *point dot,* incomplete membrane expression).

12. Statistical analysis

Differences in expression were estimated by applying an unpaired Student's *t*-test for cell lines samples analyses. The Wilcoxon's signed-rank test was applied to compare mRNA expression in tumour versus normal tissue and the non-parametric Mann-Whitney *U* test was used for comparing two different groups. The Spearman rho was calculated to find a correlation between 2 variables. A *p*-value< 0,05 was considered statistically significant. Graphs and statistical analyses were executed in Prism v6.0 (GraphPad Software, Inc.) or in SPSS Statistics v22.0 (IBM SPSS, Inc).

CHAPTER 4

RESULTS

The association of *Dies1* gene expression to differentiation programs namely, ESC differentiation and EMT/MET (group preliminary results), lead us to study this gene in a cancer model since cancer cells tend to loose differentiation along with tumour progression. Gastric cancer was the model chosen to perform this study.

Therefore, the first goal of this work was to characterize *Dies1* expression in a panel of gastric cancer cell lines and find the possible regulatory mechanism that may explain the different expression levels of *Dies1* in gastric cancer. Also, for a better characterization of *Dies1* expression in gastric cancer, its mRNA levels were determined in a series of 30 normal-tumour paired samples.

The second aim of this work was based on previous results achieved in our group in an *in vitro* EMT/MET model that revealed high levels of *Dies1* expression in epithelial states and a decrease of its expression in mesenchymal states. E-cadherin loss or down-regulated expression is a characteristic marker in EMT process that is frequently accompanied by an up-regulation of N-cadherin expression. The switch of this cadherin proteins are considered a *hallmark* of EMT and have been described in gastric cancer by Lei, et al. (85). Taking these considerations, to accomplish the second goal: “study the possible relation of *Dies1* expression with epithelial or mesenchymal markers”, the previously gastric cancer series characterized for *Dies1* expression was used for *CDH1* (encode E-cadherin protein) and *CDH2* (encode N-cadherin protein) mRNA levels determination. Also the E-cadherin and N-cadherin protein expression was characterized by immunohistochemistry in the same cases used for the mRNA expression study.

Preliminary results

Dies1 gene was studied in our group for the first time in an *in vitro* EMT/MET model. In this model, EpH4 mouse mammary epithelial cells (E cells) acquire a mesenchymal (M cells) phenotype after stimulated with the TGF-β1 cytokine. This phenotype was reverted after the stimulus cession and cell re-acquired the epithelial-like characteristics (“reversion E cells”, RE cells). The study of transcription variations during the EMT/MET process using the *Whole Transcriptome Sequencing,* revealed a variation of *Dies1* expression. This gene was down-regulated when cells lost their epithelial differentiation, M cells, and its mRNA levels increased in RE cells that re-acquire an epithelial-like phenotype (**figure 5A**). The *Dies1* up-regulation, in the mouse EMT/MET model was accompanied by a

decrease of the methylation status of *Dies1* promoter region, suggesting that this can be a possible expression regulatory mechanism in this model (**figure 5B**).

Besides the EMT/MET model, a bioinformatics analysis of different gastric cancer series revealed a down-regulation of *Dies1* in gastric cancer. Also, a preliminary characterization of *Dies1* expression was performed in a series of 13 primary gastric cancer samples (4 diffuse, 8 intestinal and 1 mixed type) in comparison with a pool of non-neoplastic gastric samples (n= 10) from an available IPATIMUP dataset. In these analyses, 12 of the 13 gastric cancer samples had a decrease of *Dies1* mRNA levels, with a significant variation of *Dies1* expression ($p < 0.05$) between the normal stomach pool and the gastric cancer samples (**figure 5C**).

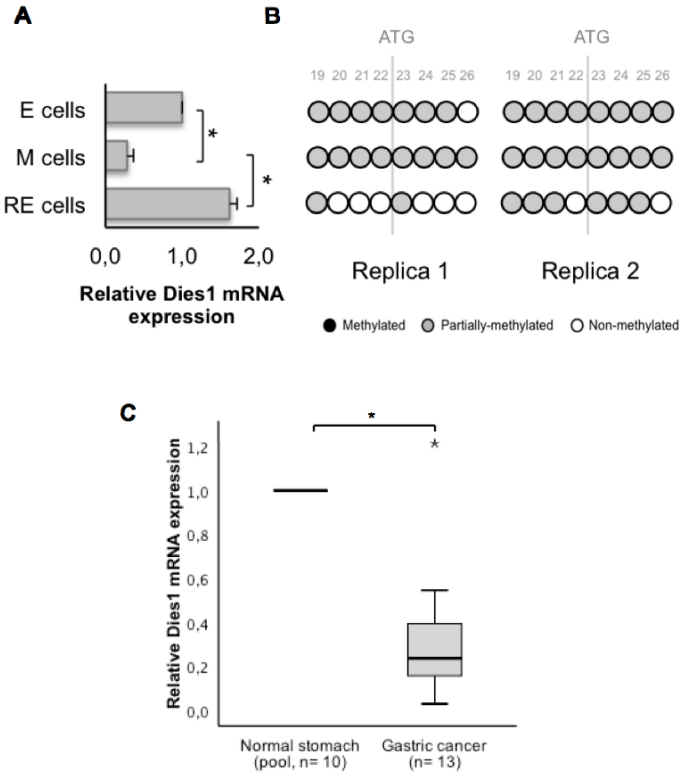


Figure 5 – Preliminary results of *Dies1* expression and promoter methylation status in EMT/MET *in vitro* model and *Dies1* mRNA levels in gastric cancer A. *Dies1* expression in EMT/MET process. mRNA levels of *Dies1* in E, M and RE cells. Expression levels were relative to the E cells mRNA level and normalized to the corresponding *GAPDH* mRNA levels. Data are present as means ± SD of duplicates **B.** *Dies1* mouse promoter methylation status in E, M and RE cells (n= 2) for CpG sites 19-26 (chr10: 59809756-59809808) Black, grey and white circles outlined at black represent methylated, partially-methylated and non-methylated CpG sites, respectively. **C.** *Dies1* expression analysed in 13 gastric cancer samples in comparison to a pool of normal stomach samples (n= 10). mRNA level was normalized with the corresponding *18S* mRNA level. A. Unpaired Student's *t*-test, B. and Non-parametric Mann-Whitney U test was used, * $p < 0.05$.

1. Dies1 expression characterization and regulatory mechanisms

1.1. Gastric cancer cell lines display down-regulation of *Dies1* mRNA levels

In present work, a panel of 7 gastric cancer cell lines namely, MKN45, IPA220, KATO III, AGS, NCI N87, GP202 and MKN28 were cultured in the same conditions and, at 70-100% of confluence, DNA and RNA (including small RNAs) extraction were performed. We characterized the *Dies1* expression using the quantitative real time PCR (qRT-PCR) technique for all seven parental gastric cancer cell lines in 3 biological replicas. The commercial RNA from normal gastric mucosa was used for normal comparison.

The majority of cell lines (6/7) revealed a significant decrease of *Dies1* expression (p<0.05) when compared with the normal counterpart. Indeed the *Dies1* mRNA level in AGS cell line was almost undetected by qRT-PCR. The NCI N87 cell line demonstrated a high heterogeneity in *Dies1* expression, among replicates, with a range between 0.4 and 2.3 fold change (**figure 6**).

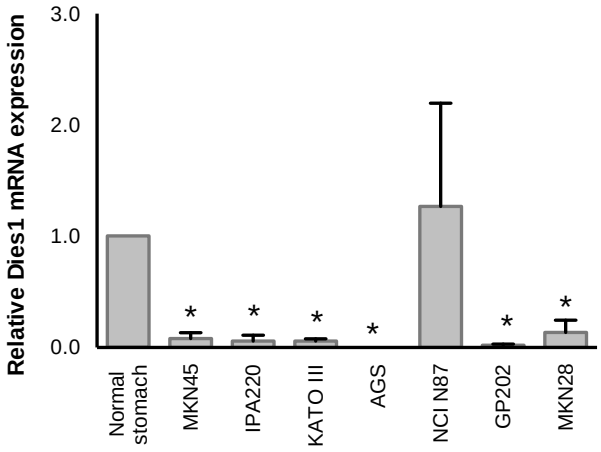


Figure 6 – Down-regulation of *Dies1* mRNA expression in gastric cancer cell lines. qRT-PCR relative quantification of *Dies1* expression in gastric cancer cell lines (3 biological replicas). mRNA expression was relative to the normal gastric mucosa (RNA commercially available) and normalized using human *GAPDH* endogenous control. Data are present as means ± SD of triplicates. Unpaired Student's *t*-test, * $p < 0.05$.

1.2. Regulatory mechanisms that may lead to *Dies1* down-regulation

Taking into account the previous results that reveal an overall down-regulation of *Dies1* in gastric cancer cell lines, and in a series of gastric cancer samples, our subsequent objective was to find a expression regulatory mechanism that could lead to a decrease of *Dies1* mRNA levels in this cancer model.

1.2.1. Regulatory mechanisms at DNA level: *Dies1* gene point-mutations

Genetic alterations that include point mutations, frameshift deletions and LOH are frequently found in gastric cancer and can lead to inactivation of tumour suppression genes. The continuous search for new driver gene alterations and the recent advances in molecular research has the potential to provide an elucidation or discovery of genes inactivated in gastric cancer. In a recent study using the whole-genome sequencing of 100 tumour-normal pairs for identify a possible new driver mutations in gastric cancer, 2 non-synonymous base changing alterations were found in *Dies1* gene sequence (heterozygosity alteration, C→T, protein coordinate: L213F; and heterozygosity alteration, C→A, protein coordinate: N307K) (130).

Based on these findings, and once the genetics alterations have an important role in carcinogenesis, a screening for *Dies1* mutations were performed in the gastric cancer cell lines used in this study. To achieve this, we sequenced the coding region of *Dies1* gene (7 exons), the 5'UTR sequence and a partial sequence of the 3'UTR that contains the predicted target site for miR-125a-5p. Nine pairs of primers were designed to cover all this sequence (**figure 7A**). The multiplex PCR technique was performed in 2 reactions in order to amplify 6 amplicons of the gene (the initial sequence of exon 2, exon 3, 4, 5, and 6 and the partial sequence of 3'UTR). However, due the unspecific products formed, 3 sequences (exon 1, final sequence of exon 2 and exon 7) were independently amplified. The gel band extraction and purification was performed before sequencing the exon 7, due the presence of an unspecific band after PCR amplification (**figure 7B**).

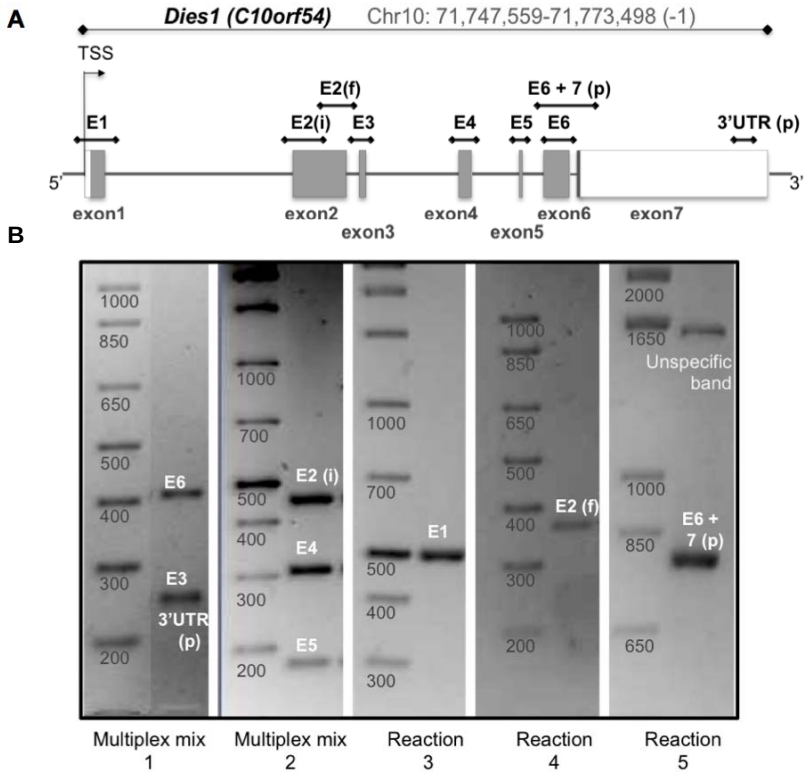


Figure 7 – Representation of *Dies1* gene sequence and electrophorese gel with the amplicons for *Dies1* mutations screening. A. Schematic representation of *Dies1* gene sequence and PCR amplicon localization. White and grey squares represent the UTR or translated sequence, respectively. Grey line represents the intron region and black lines correspond to specific amplicon localization. **B.** Representation of amplicons in the electrophorese gel for the different reactions realized to *Dies1* gene sequencing. Amplicons size: E1: 502bp, E2(i): 456bp; E2(f): 360bp; E3: 250bp; E4: 306bp; E5: 175bp; E6: 400bp; E6 + 7(p): 792bp; 3'UTR(p): 243bp. TSS, transcription start site; E, exon; i, initial, f, final; p, partial.

All PCR products were independently sequenced. Base substitutions were found when the gene sequence was compared to its reference sequence, provided by the Ensemble database. Two missense substitutions were found, one in exon 1 (C**T**G → C**C**G, located at chr10:71773420; protein coordinate: L7P) of the IPA 220 and NCI N87 cell line and the other in exon 3 (GA**T** → GA**G,** located at chr10:71760875; protein coordinate: D187E) in all the cell lines studied. Further analysis revealed that this 2 alterations were already reported as single nucleotide polymorphism (SNP), with an allele frequency higher than 1%, according to the 1000 Genomes Project Phase 1. Others 3 base change alterations, with a synonymous consequence and also classified as SNP alterations were detected. In all cell lines analysed, no alterations were found in 3'UTR predicted target site for miR-125a-5p, and this region remain conserved for possible interaction with this microRNA.

All of the variations found in *Dies1* sequencing of the 7 cell lines analysed are resumed in **table 6**.

Table 6 – Genetics variations in *Dies1* gene sequence of gastric cancer cell lines.

Samples	Exon	Alleles	Allele change	Variation class and reference	Allele frequencies[1]	Protein residue change	Consequence type
MKN45	3	(G/G)	GA**T** ⇒ GA**G**	SNP rs3747869	**T**: 12%; **G**: 88%	D [Asp] ⇒ E [Glu]	Missense
IPA220	1	(C/C)	C**T**G ⇒ C**C**G	SNP rs3747862	**T**: 70%; **C**: 30%	L [Leu] ⇒ P [Pro]	Missense
	2	(A/A)	GA**G** ⇒ GA**A**	SNP rs3747867	**G**: 79%; **A**: 21%	E [Glu] ⇒ E [Glu]	Synonymous
	3	(G/G)	GA**T** ⇒ GA**G**	SNP rs3747869	**T**: 12%; **G**: 88%	D [Asp] ⇒ E [Glu]	Missense
KATO III	2	(G/A)	GA**G** ⇒ GA**A**	SNP rs3747867	**G**: 79%; **A**: 21%	E [Glu] ⇒ E [Glu]	Synonymous
	3	(G/G)	GA**T** ⇒ GA**G**	SNP rs3747869	**T**: 12%; **G**: 88%	D [Asp] ⇒ E [Glu]	Missense
AGS	2	(C/T)	GA**C** ⇒ GA**T**	SNP rs3747866	**C**: 82%; **T**: 18%	D [Asp] ⇒ D [Asp]	Synonymous
	3	(G/G)	GA**T** ⇒ GA**G**	SNP rs3747869	**T**: 12%; **G**: 88%	D [Asp] ⇒ E [Glu]	Missense
NCI N87	1	(T/C)	C**T**G ⇒ C**C**G	SNP rs3747862	**T**: 70%; **C**: 30%	L [Leu] ⇒ P [Pro]	Missense
	2	(T/T)	GA**C** ⇒ GA**T**	SNP rs3747866	**C**: 82%; **T**: 18%	D [Asp] ⇒ D [Asp]	Synonymous
	3	(G/G)	GA**T** ⇒ GA**G**	SNP rs3747869	**T**: 12%; **G**: 88%	D [Asp] ⇒ E [Glu]	Missense
	7	(C/T)	GT**C** ⇒ GT**T**	SNP rs114278565	**C**: 97%; **T**: 3%	V [Val] ⇒ V [Val]	Synonymous
GP202	3	(G/T)	GA**T** ⇒ GA**G**	SNP rs3747869	**T**: 12%; **G**: 88%	D [Asp] ⇒ E [Glu]	Missense
MKN28	2	(C/T)	GA**C** ⇒ GA**T**	SNP rs3747866	**C**: 82%; **T**: 18%	D [Asp] ⇒ D [Asp]	Synonymous
	2	(A/A)	GA**G** ⇒ GA**A**	SNP rs3747867	**G**: 79%; **A**: 21%	E [Glu] ⇒ E [Glu]	Synonymous
	3	(G/G)	GA**T** ⇒ GA**G**	SNP rs3747869	**T**: 12%; **G**: 88%	D [Asp] ⇒ E [Glu]	Missense

[1] allele frequency according the 1000 Genomes Project Phase1;
Abbreviations: SNP, single nucleotide polymorphism; Asp, aspartic acid; Glu, glutamic acid; Leu, leucine; Pro, proline; Val, valine.

1.2.2. Regulatory mechanisms at DNA level: *Dies1* promoter methylation

Besides genetic alterations, epigenetics alterations have also an important role in gastric carcinogenesis. DNA methylation is one of the widely characterized epigenetics modifications and characteristic changes have been reported in many diseases such cancer (131). This mechanism comprises an addition of a methyl group in the fifth carbon position of a cytosine residue in a CpG dinucleotide. The clusters of CpG dinucleotide (CpG islands) are frequently located at transcription start sites (promoter), and its methylation is generally association with closed chromatin state and inhibition of promoter activity (132, 133).

In gastric cancer, diverse genes have been shown to have an association between expression down-regulation and promoter methylation. Taking in account the relevance of this regulatory mechanism in cancer and the preliminary results that revealed a possible relation between *Dies1* expression and the status of promoter methylation in the

EMT/MET model, the promoter methylation was studied as a possible mechanism leading to down-regulation of *Dies1* gene in gastric cancer cell lines.

The search for the possible presence of a CpG island in promoter region of human *Dies1* gene was further accomplish in our group, by a bioinformatics analyses. This search detected a possible CpG island in *Dies1* transcription start site and we analysed 50 *CpG* sites located at chr10: 71773310-71773801, using the sodium bisulfite genome sequencing method. The sodium bisulfite treatment leads to a deamination of non-methylated cytosine to uracil that is replaced by thymine after PCR amplification. Methylated cytosine remains intact (without base change). The DNA methylation status was interpreted by comparison the sequencing results with the original DNA sequence, in this way the presence of a cytosine indicated the existence of a methylated nucleotide, and the presence of a converted thymine indicated a non-methylated nucleotide. The presence of both C- and T- peak was considered as a partially methylated CpG site (**figure 8A**).

The analyses interpretation of the promoter methylation status results revealed that the AGS cell line presented a high number of CpG sites methylated, and the GP202 cell line had the CpG island predominantly partially methylated (**figure 8B**). These promoter methylation results were associated with down-regulation of *Dies1* mRNA levels, suggesting that this may be a regulatory mechanism of *Dies1* expression in these 2 cell lines. Also the NCI N87 cell line, that did not have a significant variation of *Dies1* expression, presented an unmethylated CpG island (**figure 8C**). Despite these results, the remaining cell lines analysed did not display a methylation profile of CpG island in *Dies1* promoter region but presenting a down-regulation of *Dies1* mRNA levels, suggesting that others mechanism could be leading to a decrease of gene expression.

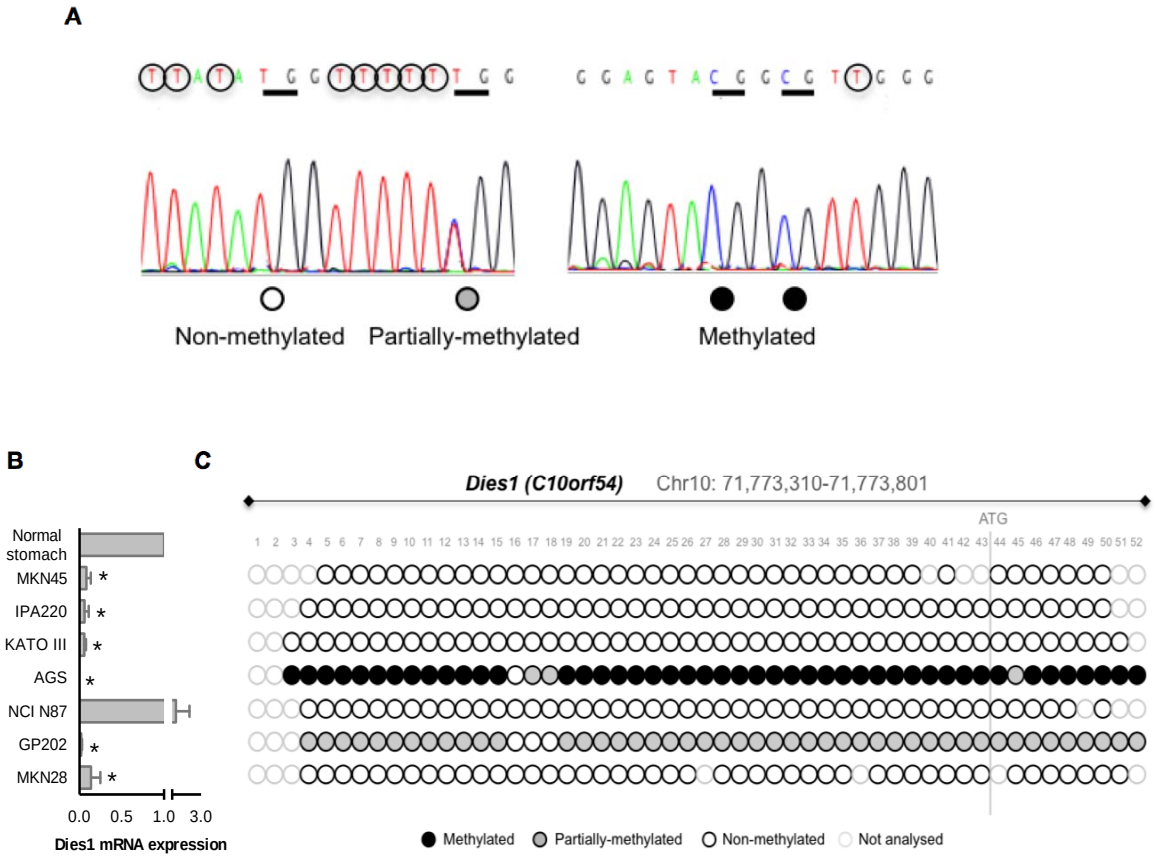


Figure 8 – *Dies1* promoter methylation status in gastric cancer cell lines. A. Interpretation of the methylation sequencing electropherogram results. The figure shows a portion of *Dies1* promoter region of AGS cell line (CpG sites 16, 17, 22 and 23). CpG sites are underlined at black and cytosines in non-CpG sites converted to thymine are indicated with a circle. CpG site with the cytosine converted to thymine represented a non-methylated site, the presence of 2 peaks (T- and C- peak) indicates partial methylation, and the existence of the cytosine in electropherogram result indicates the presence of 5mC in the genome. **B.** *Dies1* mRNA expression variation in gastric cancer cell lines and **C.** the corresponding promoter methylation status. Each horizontal row of circles represents one cell line analyses of bisulfite-treated DNA in 50 CpG sites of *Dies1* promoter region (chr10: 71773310-71773801). Black, grey and white circles outlined at black represent methylated, partially-methylated and non-methylated CpG sites, respectively. White circles outlined at grey correspond to CpG sites not analyzed. Unpaired Student's *t*-test, * $p < 0.05$.

1.2.3. Regulatory mechanisms at RNA level: regulation by miR-125a-5p

Taking in consideration the regulatory mechanisms previously studied for this gene, the gene regulation by microRNAs, namely by miR-125a-5p was also studied in gastric cancer cell lines.

Parisi et al. (18) reported that mouse *Dies1* 3'UTR sequence has a predicted target site for miR-125a. In that work, it was also proved that the overexpression of miR-125a lead to a down-regulation of Dies1 protein levels. To estimate if the human homologue miR-125a-5p, can be a possible target for human *Dies1* transcript, we used 2 approaches: first of all, using the Ensemble database, we uncovered that the human *Dies1* 3'UTR sequence contains the predicted target-site for miR-125a-5p, and the sequence of this miRNA, was

conserved between mouse and human species (**figure 9A**). In a second analysis, using the database on predicted and validated miRNA targets, miRWalk, we confirmed that the human *Dies1* mRNA 3'UTR region has a predicted seed region for miR-125a-5p.

Taking these analyses in consideration, and in order to find if there was a relationship between *Dies1* and miR-125a-5p expression, the level of this microRNA was measured by qRT-PCR.

This analysis revealed that 3 of the 7 cell lines studied, namely AGS, NCI N87 and MKN28 had an increase of miR-125a-5p expression ($p > 0.05$) compared with normal stomach mucosa. The others 4 cell lines, 3 (MKN45, IPA220 and GP202) had a decrease of expression of this miRNA compared with normal stomach mucosa ($p < 0.05$), and KATO III had only a slight reduction of miR-125a-5p expression compared with normal stomach mucosa ($p = 0.017$). AGS and MKN28 cell lines had an increase of the miR-125a-5p expression ($p = 0.219$; $p = 0.316$, respectively) concomitant with the down-regulation of *Dies1* mRNA levels, and the cell line KATO III had a higher expression of miR-125a-5p when compared to *Dies1* expression (**figure 9B**).

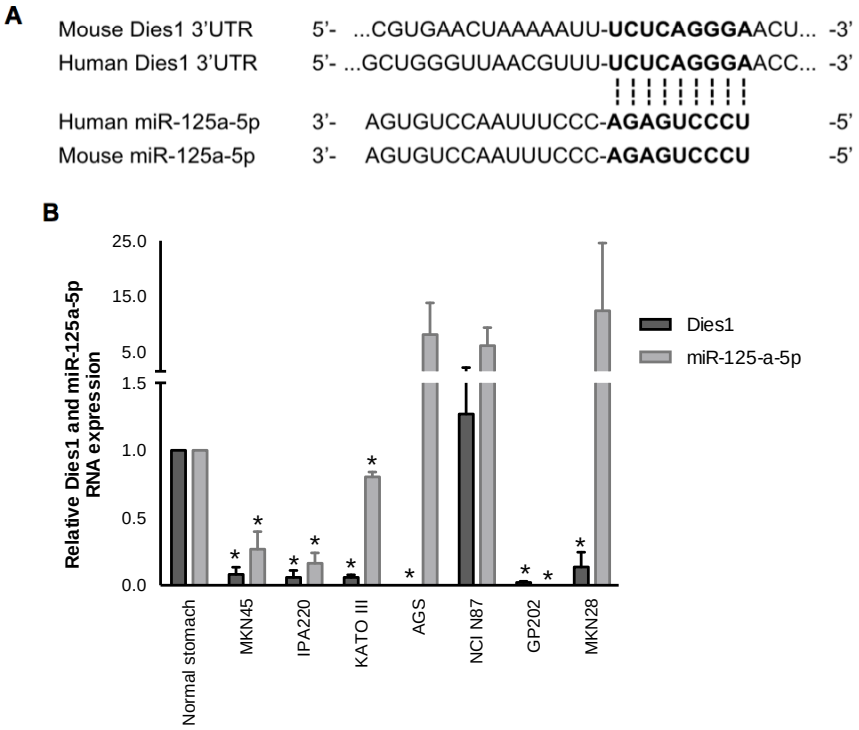


Figure 9 – miR-125a-5p expression correlation with *Dies1* gene expression. A. Bioinformatics predicted target site of miR-125a-5p in 3'UTR of *Dies1* in human and mouse species. The seed region (represented at bold) and the miR-125a-5p sequence are conserved regions between mouse and human species. **B.** qRT-PCR relative quantification of *Dies1* (results represented in figure 6) and miR-125a-5p expression levels in gastric cancer cell lines. The miR-125a-5p expression levels were normalized using the endogenous control *RNU48* and also normalized to the expression levels observed in commercial RNA from normal gastric mucosa. Data are present as means ± SD of 2 biological replicates. Statistical analyses correspond to the comparison of gene expression with correspondent expression in normal stomach. Unpaired Student's *t*-test, * $p < 0.05$.

1.3. Human gastric cancer and *Dies1* expression

For a better characterization of *Dies1* expression in gastric cancer a series (n= 30) from Portuguese patients were obtained from Hospital S. João's tissue tumour bank. The DNA and RNA extraction was completed from frozen gastric cancer samples and their normal sample pair. *Dies1* mRNA levels were measure by qRT-PCR. Due to technical problems, only 25 cases were eligible for *Dies1* expression analyses (intestinal type n= 13, diffuse type n= 12).

The analysis of the overall *Dies1* expression revealed a statistically significant difference (*p*= 0.016) between normal tissues and tumour samples, where the tumour tissue had an increase of *Dies1* expression (**figure 10A** and **B**).

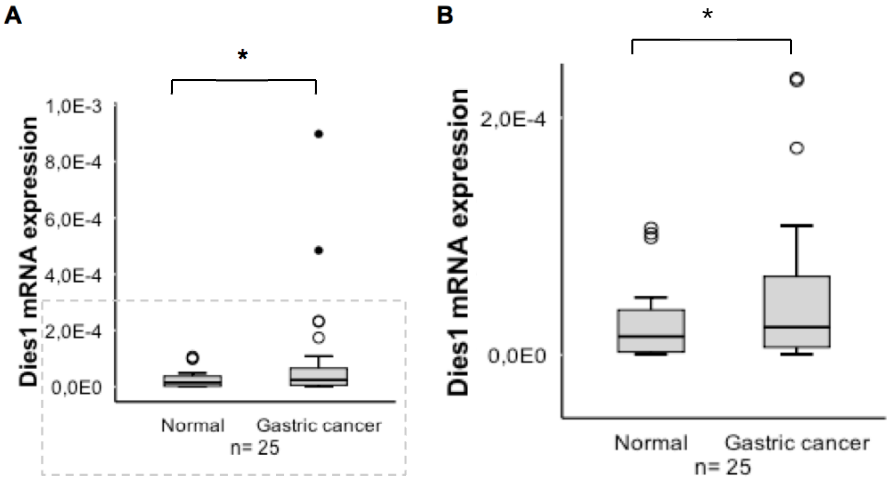


Figure 10 – Expression quantification of *Dies1* in gastric cancer samples. A. Box plots represent the mRNA expression levels of *Dies1* relative to *18S* in 25 normal gastric mucosa and gastric cancer samples. Statistically Wilcoxon Signed Ranks test was performed to obtain *p* value. * $p < 0.05$. **B.** Zoom of graphic A.

Subsequently we evaluated the *Dies1* expression in the 2 different histological subtypes following the Lauren´s classification: intestinal and diffuse type. An increased expression of *Dies1* in intestinal tumours was observed when compared with normal pairs (*p*= 0.023), and the diffuse subtype showed the same trend without statistical significance (**figure 11A** and **B**).

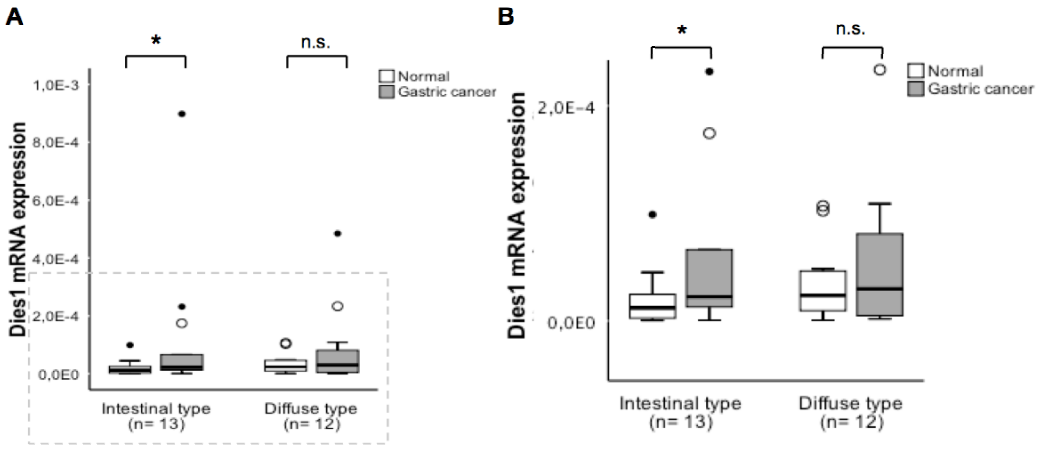


Figure 11 – *Dies1* expression in gastric cancer histological subtypes. A. Box plot represent *Dies1* mRNA expression levels relative to *18S*, subdivided by histological subtypes. **B.** Zoom of graphic A. Statistically Wilcoxon Signed Ranks test was performed for paired samples (normal/tumour). * $p < 0.05$; n.s., not-significant.

The individual analyses of *Dies1* mRNA levels compared with the normal counterpart reveal that, contrary to the preliminary results, 14 of 25 (56%) gastric cancer samples had an increase of *Dies1* expression when compared with the normal tissue, higher than 1.5 fold change. About 32% (8/25) of these cases belong to the intestinal histological subtype and 24% (6/25) belong to the diffuse type. Six of 25 (24%) tumour samples had a decreased expression similar to the previous analysis in cell lines and in the first series of gastric cancer samples, 4 of these cases were classified as diffuse type. The remaining 5 of 25 (20%) tumour cases exhibited only a slight variation of *Dies1* mRNA levels (**figure 12**).

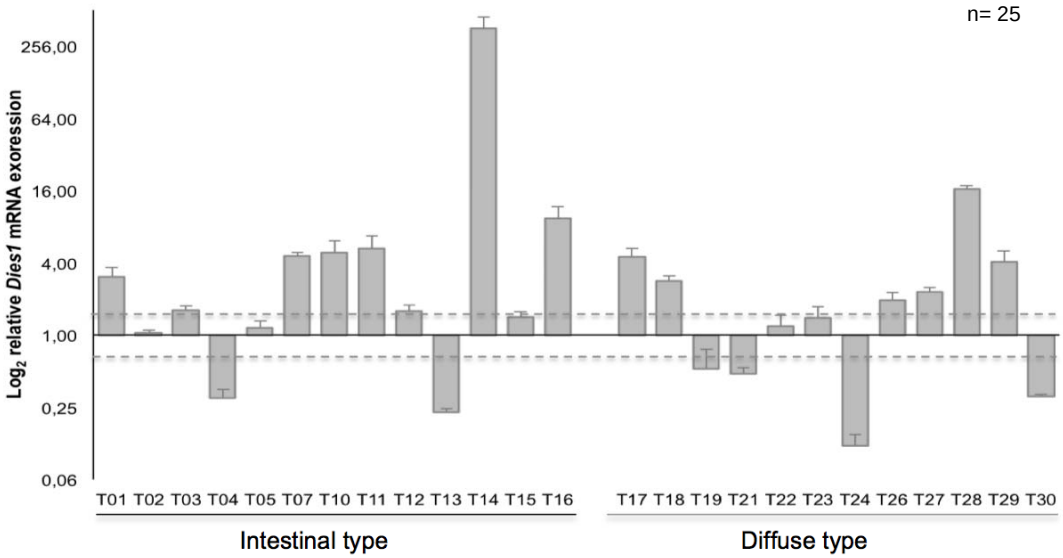


Figure 12 - Relative *Dies1* expression in tumours vs. corresponding normal (n= 25). *Dies1* mRNA level was normalized with the corresponding *18S* mRNA level. Data are present as means ± SD of technical duplicates or triplicates. The dashed lines correspond to the limits in which the expression is considered increased (1.5 fold higher) or decreased (0.66 fold lower) relative to corresponding normal.

Focusing on tumours with a down-regulation of *Dies1* expression in comparison with the corresponding normal and, similar to the results obtained for cell lines, we analysed one of the mostly likely expression regulatory mechanism: promoter methylation. In order to do that, the DNA of the 6 tumour samples with down-regulation of *Dies1* expression was treated with sodium bisulfite. Also, as a negative control a normal DNA sample was bisulfite converted. Bisulfite treatment of AGS cell line DNA was used as technical positive control. None of the 6 tumours with down-regulation of *Dies1* expression, and similarly to the normal sample, presented CpG island methylation (**figure 13**), suggesting that other regulatory mechanisms may be responsible for the alteration of *Dies1* gene expression.

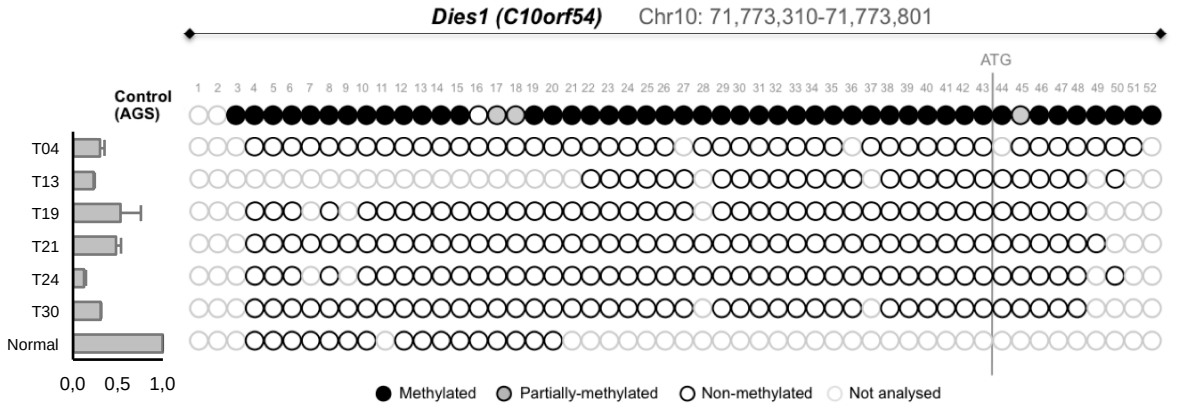


Figure 13 – Promoter methylation status of gastric cancer tumours with a decrease of *Dies1* expression relative to normal counterpart. AGS cell line functioned as technical positive control and normal stomach was used as a methylation negative control. Each horizontal row of circles represents one tumour sample analyzed for 50 CpG sites of *Dies1* promoter region (chr10: 71773310-71773801). Black, grey and white circles outlined at black represent methylated, partially-methylated and non-methylated CpG sites, respectively. White circles outlined at grey correspond to CpG sites not analyzed.

As methylation does not explain *Dies1* loss in these gastric cancers, it remains to be further analysed the expression of miR-125-5p in the gastric cancer series.

1.4. Dies1 protein characterization

In order to characterize the Dies1 protein expression on gastric normal tissue, with the main goal of understanding the impact of Dies1 down-regulation at the RNA level, IHC optimization was performed in paraffin-embedding tissues, with antibodies against Dies1.

Dies1 IHC optimization was performed in a TMA with mouse tissues and in normal human gastric mucosa. The TMA was used for better characterization of this protein in different normal tissues. The TMA construction was also performed for other antibody optimizations.

To achieve Dies1 antibody optimization, we performed different experiments using for that different antigen retrieval approach: different methods (heat and proteolytic digestion

methods) and different buffer solutions (EDTA and citrate buffer). Also, different antibody concentrations and incubation times were tested (**table 5**, **material and methods chapter**). Besides the apparent specific staining accomplished in mouse tissues, in human samples this staining was heterogeneous with different staining patterns even after optimization of the best antibody condition (R&D Systems, clone 730802; antigen retrieval: IHC-Tek Epitope Retrieval Steamer Set, 40 min, Citrate Buffer; antibody condition: dilution 1:100 at overnight, 4ºC; **figure 14** and **15**). Predominantly in human samples, but also in mouse samples, IHC results, in same conditions, and in sequential tissue sections, were variable and not trustable (**figure 14**).

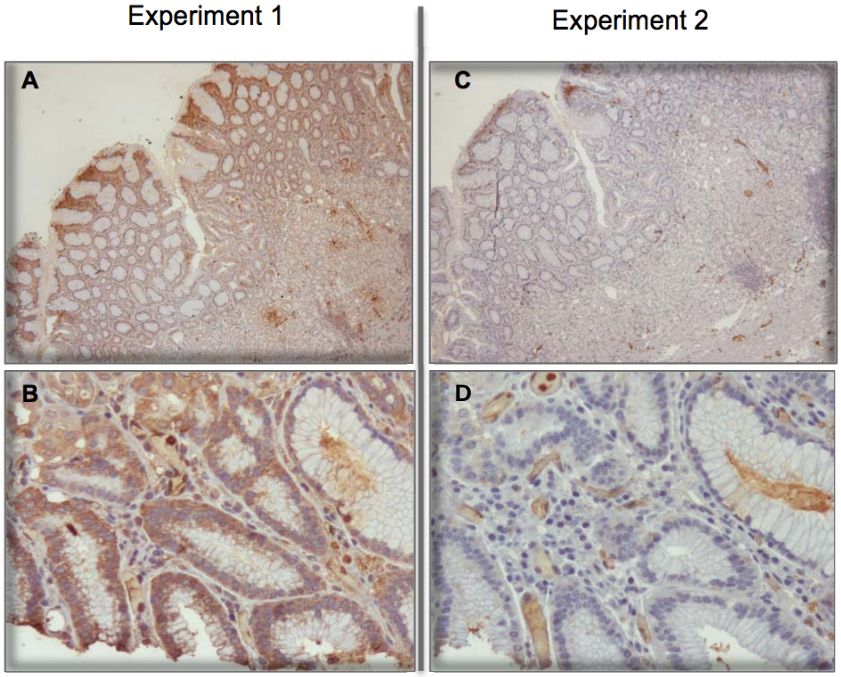


Figure 14 – Dies1 immunohistochemistry staining in normal human gastric mucosa. The staining was performed in 2 independent experiments using the same block sections and following the same protocol conditions (optimization test 1, table 5). **A.** and **B**. In experiment 1 the antibody seems to stain the gastric glands and some stromal cells. **C.** and **D**. In experiment 2 there are no staining in glandular, only some staining was seen in stromal cells. A. and C. original magnification ×40; B. and D. original magnification ×200.

Besides this technical problem, the Dies1 protein, in mouse, seems to be present in different tissues, mainly in muscle, gastric mucosa, spleen, colon and pancreas with a cytoplasmic/membrane staining (**figure 15**). However this results should be further confirmed.

The acquisition of a new antibody did not solve the immunostaining problems once no staining was obtained neither by immunohistochemistry, immunocytochemistry or western blot techniques. The acquisition or production of a new antibody is likely necessary for further characterization of Dies1 protein in different tissues, in normal gastric mucosa and in gastric cancer.

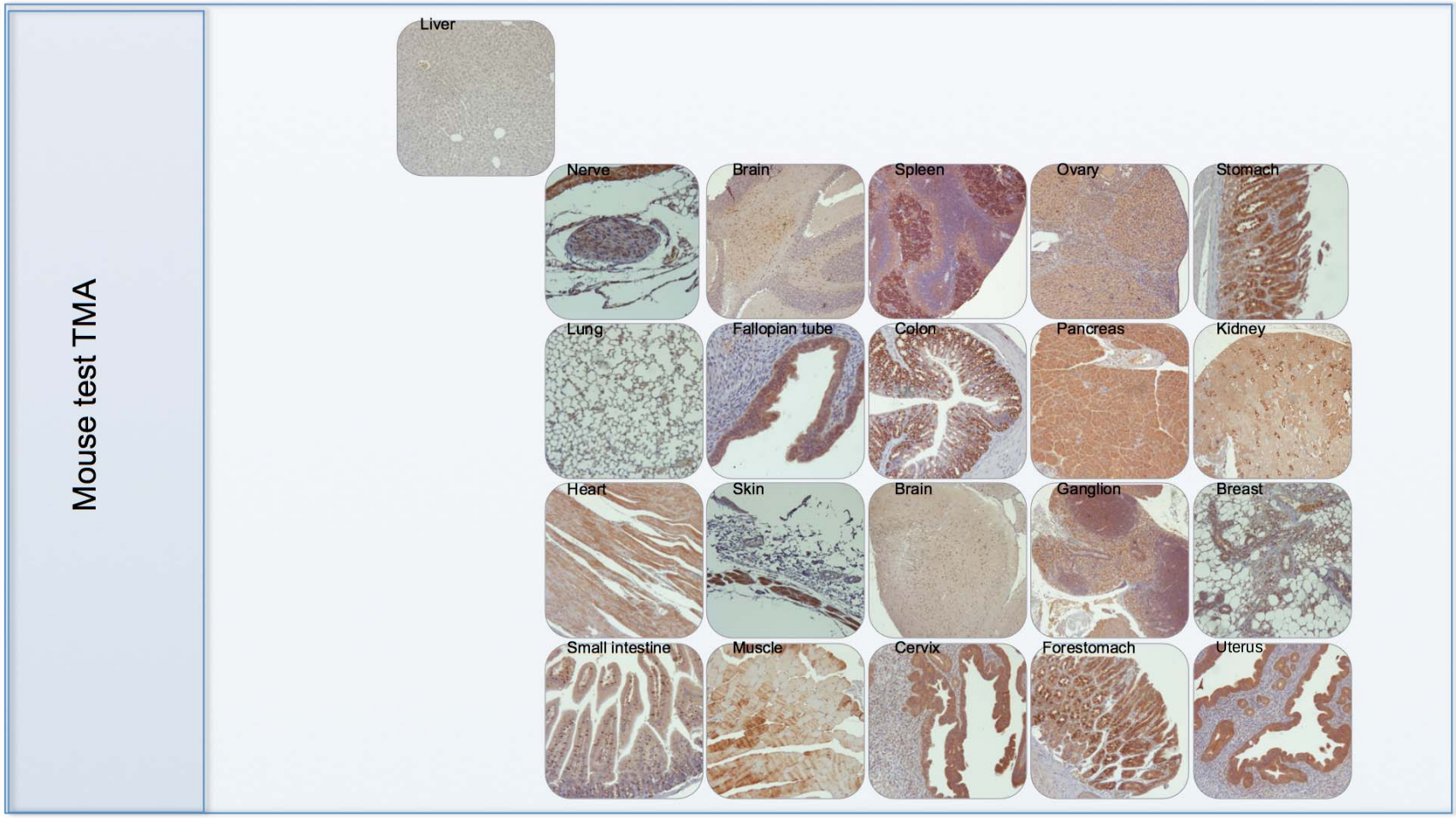


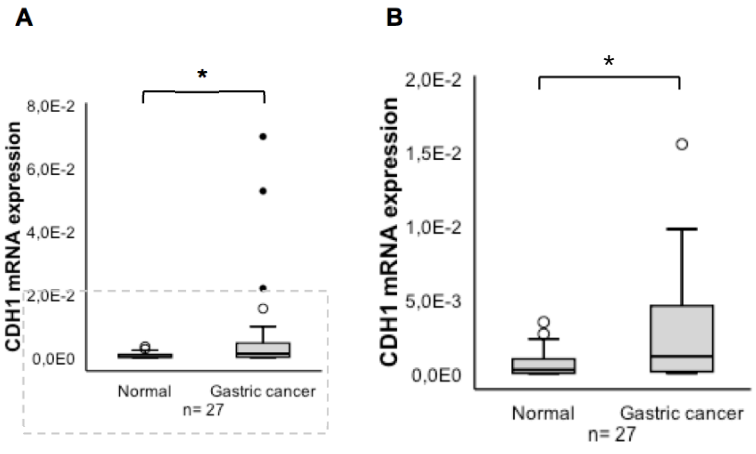
Figure 15 – TMA mouse tissue immunostaining for Dies1 protein. Dies1 protein seems to have cytoplasmic and membrane localization. Nerve, Stomach, Lung, Muscle at original magnification×100; Remaining tissues at original magnification×40. **Positive tissues:** Nerve, Brain, Spleen, Ovary, Stomach, Fallopian tube, Colon, Pancreas, Kidney, Heart, Brain, Ganglion, Breast, Small intestine, Muscle, Cervix, Forestomach Uterus. **Negative tissues**: Liver, Lung, Skin.

2. *Dies1* mRNA expression correlation with epithelial and mesenchymal markers

The analyses of *CDH1* and *CDH2* expression had the main objective of evaluating the expression correlation between *Dies1* and an epithelial and a mesenchymal marker (*CDH1* and *CDH2* respectively), in our gastric cancer series. The series was not completely characterized for the 3 gene studied (*CDH1*, *Dies1* and *CDH2)* due some technical problems, mostly related with the RNA quality.

2.1. *CDH1* and *CDH2* mRNA expression in gastric cancer

CDH1 mRNA levels were obtained for 27 gastric cancer samples (14 intestinal type, 13 diffuse type), 74.1% (20/27) of these cases presented *CDH1* up-regulation (≥ 1.5 fold change when compared with the normal pair). This increased expression correspond to 85.7% (12/14) of intestinal type tumours and 61.5% (8/13) of diffuse type tumour cases. Only 4 of 27 cases (14.8%) presented a decreased expression of this gene, when compared with the normal pair (≤ 0.66 fold change), and 3 of these were histologically classified as diffuse type. The remaining 3 tumour cases (11.1%, 3/27) exhibited only a slight variation of *CDH1* mRNA levels. As predicted by results described above, the overall difference of *CDH1* mRNA levels between normal and tumour samples was statistically significant with an increased expression observed in tumour samples ($p < 0.00$) (**figure 16A** and **B**). The evaluation of *CDH1* expression by histological subtypes also revealed an up-regulation of this gene in the intestinal tumour subtype when compared to normal pair ($p = 0.001$), in diffuse type this difference was subtle ($p = 0.101$). Data dispersion reveals a difference in *CDH1* expression between intestinal and diffuse histological tumour samples, where intestinal tumours present a higher expression than the diffuse tumour type. Besides this visual difference, these results were not statistically significant ($p = 0.073$) (**figure 16C** and **D**).



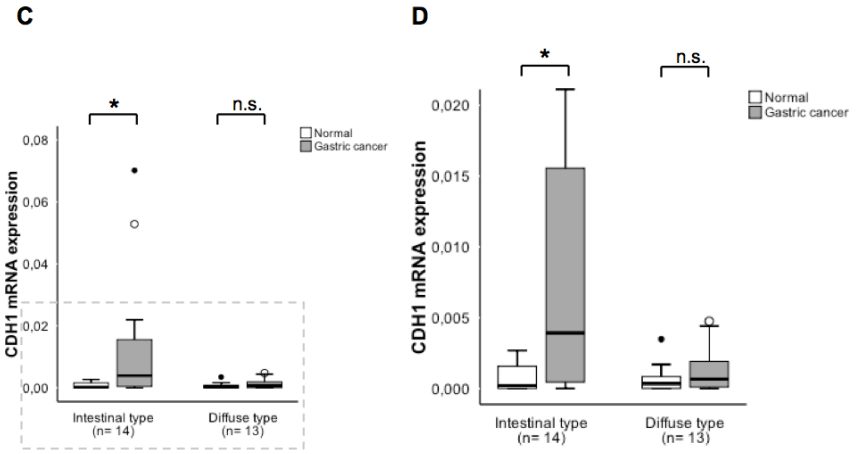
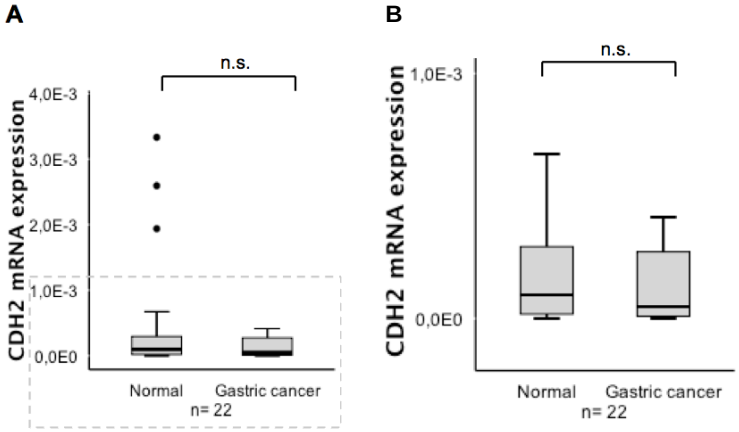


Figure 16 – Characterization of *CDH1* mRNA expression in the gastric cancer series. mRNA expression of *CDH1* relative to *18S* in 27 normal gastric mucosa and gastric cancer samples **A.** Boxplots represent the expression in normal and gastric cancer samples. Gastric cancer shown an up-regulation of *CDH1* expression (p< 0.00) when compared with normal pair **B.** Zoom of graphic A. **C.** *CDH1* expression by histological type, intestinal type n= 14, diffuse type n= 13. *CDH1* had an increase expression in intestinal tumour samples compared to their normal pair (p= 0.001), and no significant difference exists in diffuse type. **D.** Zoom of graphic C. Statistically Wilcoxon Signed Ranks test was performed for paired samples (normal/tumour). * p < 0.05, n.s., not-significant.

The analysis of *CDH2* expression was performed in 22-paired samples (9 intestinal type, 13 diffuse type). We verified that 31.8% (7/22) of tumour samples displayed an up-regulation of expression when compared to the normal counterpart, where 9.1% (2/22) of these was classified as intestinal type, and 22.7% (5/22) as diffuse type. About 45.5% (10/22) of tumour samples had a down-regulation of *CDH2* expression and 22.7% (5/22) had no expression variation. The overall mRNA expression levels did not vary between the tumour and normal counterpart samples, p> 0.05 (**figure 17A** and **B**). Also there was no difference in *CDH2* expression between the tumour and normal paired samples related with the histological type, neither between the diffuse and intestinal tumour expression, p> 0.05 (**figure 17C** and **D**).



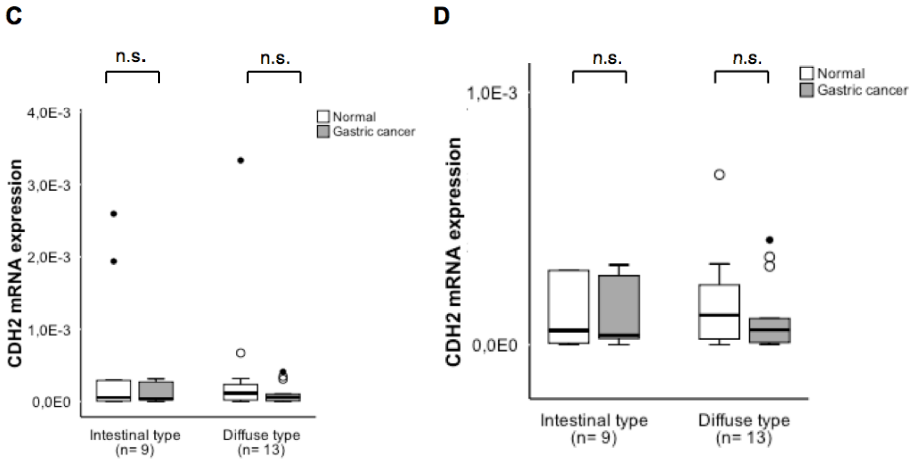


Figure 17 – mRNA expression of *CDH2* in gastric cancer tumour samples. mRNA expression levels of *CDH1* relative to *18S* in 22 normal gastric mucosa and gastric cancer samples. **A.** Boxplots represent the expression in normal and gastric cancer samples. *CDH2* overall expression showed a similar expression between normal and tumour samples. **B.** Zoom of graphic A. **C.** *CDH2* expression by histological type, intestinal type n= 9, diffuse type n= 13. **D.** Zoom of graphic C. Statistically Wilcoxon Signed Ranks test was performed for paired samples (normal/tumour). n.s., not-significant.

After characterization of 3 evaluated markers both *Dies1* and *CDH1* expression revealed a significant increased expression between normal and tumour samples. Also in both, this difference was presented in intestinal type but not in diffuse histotype.

2.2. *Dies1* expression seems to correlate with *CDH1* expression in gastric cancer samples

With the main objective to study the correlation of *Dies1* expression with an epithelial (*CDH1*) and a mesenchymal (*CDH2*) marker, the gastric tumour samples with the eligible results for the 3 genes was used for performing this analyses. This corresponded to a total of 19-paired samples (intestinal type n= 8, diffuse type n= 11). The tumour mRNA expression results were normalized for expression of the matching normal sample.

The analyses of data dispersion revealed that *CDH1* expression was higher in tumour than in normal in more than 75% of the cases, and that *Dies1* expression was higher in tumour than in normal in more than 50% of the cases. In contrast tumour samples presented a lower expression of *CDH2* in more than 50% of tumour compared to matching normal sample (**Figure 18A**).

Analysing the tumour samples by histologic subtypes, the expression of *CDH1* seems to be higher in intestinal type than in diffuse type. The same trend was verified in *Dies1* expression, and by evaluating the data dispersion, this gene seemed to have a higher expression in intestinal type in comparison to the diffuse type. No difference seems to exist between the 2 histological types concerning the *CDH2* mRNA levels (**Figure 18B**).

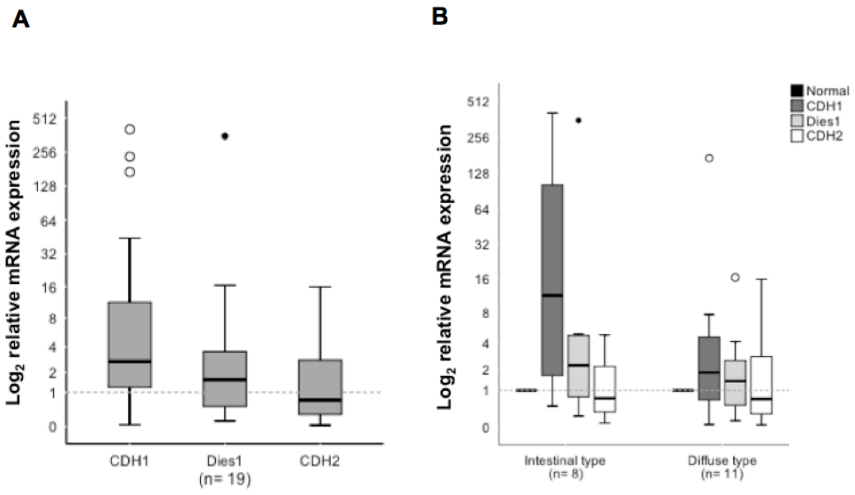


Figure 18 – *CDH1*, *Dies1* and *CDH2* expression levels variations in 19 tumours samples normalized for normal pair. A. Box plots represent mRNA expression level relative to normal pair. **B.** Relative mRNA expression divided by histological type. Intestinal tumours samples showed a higher expression of *CDH1* and *Dies1* compared to diffuse type. *CDH2* expression had the same expression in both histological subtypes.

The individual analyses demonstrated that in 17 of the 19 cases *CDH1* and *Dies1* expression levels follow the same tendency (**figure 19**, blue outlined squares). However in 3 of these cases, namely tumour case 15, 22 and 27, one of the gene variations is near to the normal expression when the other had an overexpression higher that 1.5 fold change or a decrease expression lower than 0.66 fold change in comparison with the normal paired sample. In only 2 cases *CDH1* and *Dies1* expression did not follow the same trend (tumour cases 13 and 19). In 11 cases, the 3 genes studied had the same expression tendency, in 8 of them *CDH1*, *Dies1* and *CDH2* had an overexpression higher that 1.5 (n= 4) or a decreased expression lower than 0.66 (n= 4) fold change (**figure 19**).

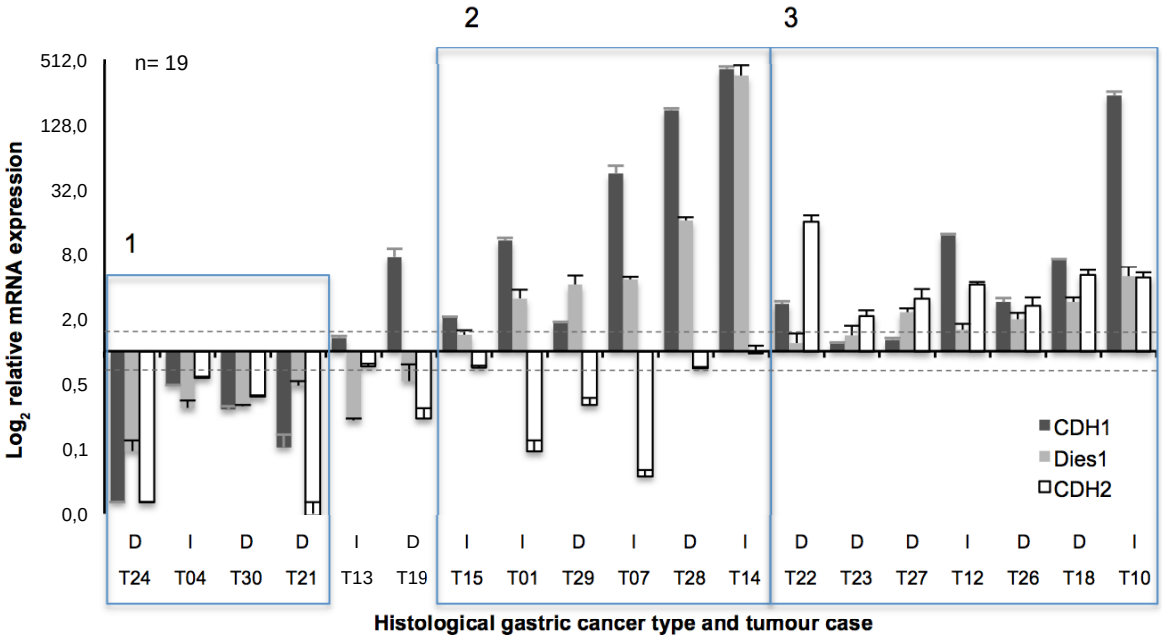


Figure 19 – Individual case analyses of *CDH1*, *Dies1* and *CDH2* relative mRNA levels variation. mRNA expression normalized for the normal pair. (1) The first 4 tumour cases had an expression down-regulation of the 3 analysed genes. Tumour case 13 and 19 were the only tumours with an opposite expression tendency of *CDH1* and *Dies1* genes. (2) Six tumour cases had an up-regulation of *CDH1* and *Dies1* expression and an opposite tendency of *CDH2* expression level. (3) Seven cases had an overexpression or a slight increase of the *CDH1*, *Dies1* and *CDH2* expression levels in tumour samples. mRNA expression levels was relative to the corresponding *18S* mRNA level. The dashed lines correspond to the limits in which the expression is considered increased (1.5 fold higher) or decreased (0.66 fold lower) relative to corresponding normal.

The above results demonstrated that most cases showed a similar mRNA expression tendency between *Dies1* and *CDH1* genes. Following these results a correlation analyses was performed for the 3 genes. This analysis revealed that there was no correlation between *CDH1* and *CDH2* mRNA levels (p= 0.180). The same results were achieved for *CDH2* and *Dies1* correlation analyses in the overall cases (p= 0.314). The correlation analyses revealed a statistically significant relationship between the expression of *Dies1* and *CDH1* markers (p< 0.01), and the dispersion data analyses lead us to hypothesise that *Dies1* and *CDH1* mRNA relative expression followed the same trend.

2.3. Immunoreactivity of E-cadherin and N-cadherin

The protein expression does not always follow the mRNA expression and the ratios between protein and mRNA are mainly determined by translation and protein degradation. (134) Additionally, even when protein is present, a high number of post translation modifications can affect the protein function. Often E-cadherin protein presents an aberrant expression in cancer due to de-localization from the cell membrane to the

cytoplasm, but the mRNA level remain the same, while the protein function becomes impaired. Taking this in account the immunohistochemistry protein characterization of our gastric cancer series was performed for E-cadherin and N-cadherin proteins. Due the heterogeneous staining, previously achieved in human tissues, it was not possible to characterize Dies1 at protein level.

The IHC technique was performed in the 19 tumours cases previously analysed at mRNA level. The immunostaining was accomplished in an automatic slide staining for ensure the same conditions in all tumour samples, and normal epithelial cells and ganglion cells functioned as positive control for E-cadherin and N-cadherin, respectively.

N-cadherin protein was not detected neither in normal epithelial mucosa neither in any of the tumour cases analysed, including the cases with an overexpression of the mRNA level when compared to the normal tissue (**figure 20A, table 7**). The specific positivity staining in internal positive control cells excluded the existence of technical problems (**figure 20B**).

E-cadherin protein staining was evaluated by comparing the expression of tumour cells with normal epithelial cells. Only 3 tumour cases, which belong to the intestinal subtype, presented a normal E-cadherin membranous expression (**figure 20C**). The remaining cases presented aberrant expression pattern such as: cytoplasmic staining, incomplete cell membranous staining, or an aberrant doted pattern (**figure 20D, E, F**). From the 5 intestinal cases with decreased intensity of the protein expression, only 1 had a concomitant decrease of mRNA expression; the other 4 cases, 2 had an increased expression and 1 had the same expression in normal and tumour sample. Also, the E-cadherin positivity, in tumour 10, was found in less than 25% of tumour cells, however mRNA levels were higher in tumour than the normal pair. In diffuse histological type, all tumour cases exhibit an abnormal pattern of E-cadherin staining, 4 tumour cases was no E-cadherin staining and 2 had expression in less than 25% of tumour cells. In these 6 cases the mRNA level only had a correlation with the protein level in 1 case (T21), the other 5 presented an up-regulation or no variation of *CDH1* expression in comparison with the normal tissue. In tumour 30, mRNA expression was decreased, but 90% of tumour cells had a normal positive staining and only 10% had an aberrant staining pattern.

The mRNA expression results of the 3 genes and the IHC results are resumed in **table 7**.

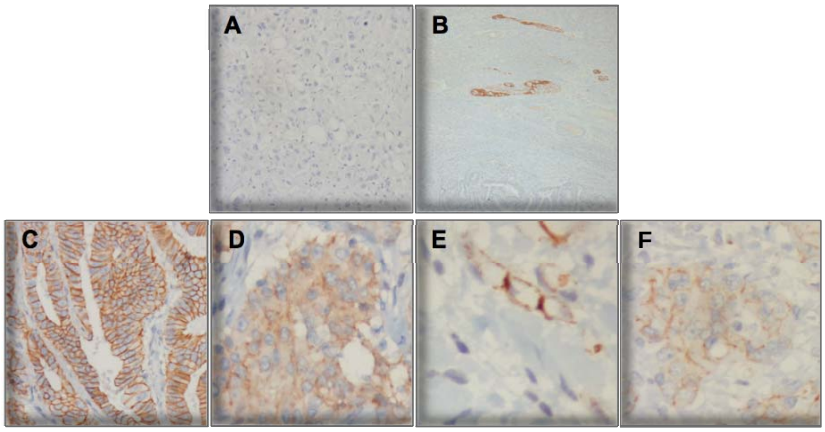


Figure 20 – N-cadherin and E-cadherin immunostaining. A. No staining was achieved for N-cadherin protein. Tumour 23, original magnification ×100 **B.** N-cadherin positive intern control, ganglion cells, Tumour 23, original magnification ×40. **C. D. E** and **F.** Different E-cadherin immunostaining pattern on gastric cancer cells: **C.** Normal pattern; Tumour 07, original magnification ×200; **D.** Cytoplasmic staining; Tumour 15, original magnification ×200; **E.** Dot point pattern mainly on cell-cell contact; Tumour 19, original magnification ×600; **F.** Incomplete membrane staining; Tumour 10, original magnification ×400.

Table 7 – Summary of mRNA and protein expression of the analysed genes: *Dies1*, *CDH1*, *CDH2*.

Tumour ID	*Dies1* expression	*CDH1* or E-cadherin expression				*CDH2* or N-cadherin expression	
	mRNA	mRNA	Protein			mRNA	Protein
			Intensity	Protein pattern	Positive cells		Positive cells
Intestinal type (n= 8)							
T01	1	1	1+	Aberrant (incomplete)	(3) > 76%	-1	0
T04	-1	-1	1+	Aberrant (incomplete)	(2) 50-75%	-1	0
T07	1	1	3+	Normal	(3) > 76%	-1	0
T10	1	1	1+	Aberrant (incomplete and heterogeneous expression)	(0) < 25%	1	0
T12	1	1	3+	Aberrant (incomplete)	(3) > 76%	1	0
T13	-1	0	1+	Normal	(3) > 76%	0	0
T14	1	1	2+	Normal	(3) > 76%	0	0
T15	0	1	1+	Aberrant (incomplete and cytoplasmic)	(3) > 76%	0	0
Diffuse type (n= 11)							
T18	1	1	2+	Aberrant (dot point)	(3) > 76%	1	0
T19	-1	1	2+	Aberrant (incomplete and dot point)	(2) 50-75%	-1	0
T21	-1	-1	0	-	(0) < 5%	-1	0
T22	0	1	1+	Aberrant (incomplete, dot point and cytoplasmic)	(0) < 25%	1	0
T23	0	0	1+	Aberrant (incomplete and dot point)	(0) < 25%	1	0
T24	-1	-1	2+	Aberrant (incomplete and dot point)	(2) 50-75%	-1	0
T26	1	1	0	-	(0) < 25%	1	0
T27	1	0	1+	Normal (Superficial cells); Aberrant (Profound cells: incomplete, dot point and cytoplasmic)	(2) 50-75%	1	0
T28	1	1	0	-	(0) 0%	0	0
T29	1	1	0	-	(0) 0%	0	0
T30	-1	-1	2+	Normal (90%), Aberrant (dot pattern, 10%)	(3) > 76%	-1	0

The mRNA values were relative to the normalized expression of genes in tumour samples: -1, <0.66 fold change; 0, values between 1.5 and 0.66 fold change; 1, >1.5 fold change. Protein evaluation: Intensity: 0, no staining, 1+, weak staining, 2+, moderate staining 3+, strong staining. Positive cells: 0, 0-25%; 1+, 26-50%; 2+, 51-75%; 3+, > 76% of positive tumour cell.

CHAPTER 5

DISCUSSION

DISCUSSION

In this thesis, we aimed to (1) characterize the *Dies1* expression in gastric cancer cell lines and tumour samples, and explore the possible expression regulatory mechanisms of this gene, and; (2) analyse the expression relationship of *Dies1* with an epithelial (E-cadherin) and a mesenchymal (N-cadherin) marker. Overall, and in relation to aim 1, we observed that *Dies1* expression is modulated differentially in gastric cancer cell lines and primary tumours, potentially due to 2 possible mechanisms, promoter methylation and regulation by miR-125a-5p. Concerning the results related with aim 2, we observed that *Dies1* expression seems to follow the expression of the epithelial marker, *CDH1*.

The contribution of Dies1 in mESC differentiation into neurons, astrocytes, cardiomyocytes and pre-adipocytes into adipocytes pointed for the important role of this gene in embryonic differentiation program (1, 5). Also in a distinct differentiation context, such as EMT/MET, *Dies1* seems to follow the epithelial cell differentiation. In EMT, cells usually lose their epithelial differentiation and characteristics and acquire a mesenchymal phenotype, this process can be reverted and cell re-acquires the epithelial characteristics (MET) (33, 36-38). In the EMT/MET *in vitro* model established in our group, *Dies1* mRNA levels accompanied the loss of epithelial differentiation with a decreased expression in cells that undergo EMT, and a re-acquisition of its mRNA levels when cells return back to an epithelial like phenotype. In this preliminary result *Dies1* seemed to have a similar behavior to a characteristic epithelial marker.

The involvement of *Dies1* in 2 differentiation programs led us to study its expression in a gastric epithelial cancer model, where cells frequently presents some degree of de-differentiation along with cancer progression.

The first approach to study *Dies1* gene in gastric cancer was analyzing its expression in a set of gastric cancer cell lines. This gene revealed a decreased expression in the majority (6/7) of gastric cancer cell lines studied. These results are consistent with the preliminary results that described a decrease expression of *Dies1* in 13 gastric cancer samples compared with a pool of normal gastric samples. Only 1 cell line evaluated did not have a decrease in the expression of this gene, and curiously this was the only cell line characterized as a well-differentiated gastric cancer cell line (126, 135). The down-regulation of *Dies1* led us to hypothesis about the possible regulation mechanism that leads to a decreased expression in gastric cancer cell lines.

Gastric carcinogenesis is a complex multistep process, and numerous studies indicate that the development and progression of gastric cancer arises via misregulation of numerous genes. The accumulation of genetic and epigenetic alterations in different proto-oncogenes and tumor-suppression genes culminates in cellular behavior modification, and increased evidence has emerged that genetic and epigenetic mechanisms acts in combination and take advance of each other during tumorigenesis (77, 89, 136). Genetic alteration, such as mutations is a frequent mechanism that leads to a down-regulation of some genes in gastric cancer, such *TP53* and *CDH1*. (50, 108, 137) Loss of heterozygosity is a common mechanism associated with biallelic inactivation, besides the genetics modifications, epigenetics alterations such as promoter hypermethylation is an important event associated with loss of gene expression (50, 137). The promoter methylation status has been described to be responsible for down-regulation of many tumour suppression and tumour related genes in gastric cancer, such *MSH2* (MutS protein homolog 2), *MLH1 (mutL homolog 1), p16* and *RUNX3*. (137). These genetics and epigenetic regulatory mechanisms cannot explain all down-regulated gene alterations in gastric cancer. Taking the example of E-cadherin, Corso, et al. described that a high percentage of cases with alterations of E-cadherin protein expression did not have structural mechanisms (mutations and LOH) neither promoter hypermethylation, suggesting the presence of different inactivating mechanisms (108). These different mechanisms can include the regulation by microRNA, transcription factors repressors, and also posttranslational mechanisms, such aberrant *N*-glycosylation and proteolytic cleavage that can result in a deregulation of E-cadherin function (50, 106, 107).

In a first approach to find the expression regulation mechanism of *Dies1*, we searched for the presence of a genetic alteration in the set of gastric cancer cell lines. In this search, we found some point alterations, classified as SNP alterations. Although we found some base changes consistent with an missense alteration, these alterations have been reported in an high frequency in the 1000 Genomes Project Phase 1 (>1%) and in both alleles, witch lead us to infer that this alterations does not likely affect the protein function. These results can exclude the presence of mutations in the coding region, as a regulatory mechanism that could explain the down-regulation of *Dies1* in this set of gastric cancer cell lines.

Comparing the results obtained by Wang, et al. with ours results, the 2 mutations previously found in gastric cancer samples were not present in any of the 7 cell lines (130). Our results, although not revealing the mechanism of inactivation of *Dies1* in gastric

cancer cell lines, allowed us to optimize the set of primers that were further used for characterize the possible presence of *Dies1* mutations in our gastric cancer series.

Traditionally, cancer was viewed as a set of diseases that are driven by accumulation of genetics alterations, although a growing body of evidences suggest that the disrupt of epigenetic regulatory mechanism are also involved in initiation and progression of cancer (136, 138). Aberrant DNA methylation was the first epigenetic mark to be associated with cancer and accumulating evidences reveal that DNA methylation plays an important role in oncogenesis (138, 139). DNA methylation refers to the addition of a methyl group at the 5 position of the cytosine ring within CpG dinucleotides. The distribution of CpG dinucleotide in human genome are not uniform, instead they are concentrated in regions of large repetitive sequences, such centromeric repeats, and in short CpG-rich DNA areas called CpG islands, that frequently overlap with transcriptional start sites (140, 141). The hypermethylation of promoter region contributes for gene repression by inhibit the binding of activator transcription factors at their recognition sequence or recruits methyl binding proteins that recruit co-repressor molecules and leading silence transcription (132, 142). Aberrant methylation pattern, hyper or hypomethylation is present in promoter region of key players in gastric cancer leading to theirs expression regulation (139, 143).

Given the close association with promoter hypermethylation and transcriptional silencing of tumour related genes, and the previous results in EMT/MET process, we studied the *Dies1* promoter methylation pattern in our set of gastric cancer cell lines.

The CpG island located at the transcription start site presented hypermethylation in AGS cell line and partially-methylation in GP202 cell line and in both cases this methylation status were negatively correlated with *Dies1* expression. The remaining 5 cell lines studied did not present a methylation pattern in the promoter region, however 4 of them present a decrease of *Dies1* expression.

In order to confirm if methylation could be a possible regulatory mechanism of *Dies1* expression we studied the *Dies1* promoter methylation status in 6 tumour cases that presented down-regulation of the expression this gene in our gastric cancer series. None of the 6 cases presented promoter CpG sites methylated, which led us to suggest that the regulation of this gene by promoter methylation is a rare event, and the decreased expression of *Dies1* can be due to the effect of a different regulatory mechanism. The methylation profile of AGS and GP202 may be a possible mechanism for the expression down-regulation of this gene or be may unrelated with gene expression. (143, 144). Further studies it will be necessary to address if this mechanism can regulate the expression of *Dies1* gene.

Considering the variety of mechanisms that can regulate gene expression, we studied gene expression regulation by microRNA (miR-125a), another mechanism already described for Dies1 protein expression regulation in mouse embryonic differentiation context (18). In previous works was reported that miR-125a directly targets *Dies1* 3'-UTR and regulates its expression. Also the overexpression of this miRNA recapitulates the effects of Dies1 suppression and blocks the mESC differentiation. In this study the authors also pointed that miR-125a controls Dies1 level through BMP4 signaling pathway, suggesting that exists a feedback loop between miR-125a, BMP4 signaling pathway and Dies1: BMP4 induces the transcription of miR-125a through phosphoSmad1 and down-regulated the Dies1 expression that leads to the decrease the BMP4 signaling transduction (18).

In our study the miR-125a-5p expression was increased relative to the normal counterpart in 3 cell lines, AGS, NCI N87 and MKN28, however only the AGS and MKN28 had a down-regulation of *Dies1* gene. Also, KATO III cell line had a higher expression of miR-125a-5p compared to *Dies1* expression, but still lower than the expression of the normal gastric tissue.

Once the miR-125a expression variation and its function as a regulatory mechanism for Dies1 expression has been related with BMP4 signaling pathway, we performed a search in the literature and found that only 4 (IPA220, AGS, GP202 and MKN28) of the 7 cell lines analyzed seem to have this signaling pathway functional. MKN45, KATO III and NCI N87 cell lines have been reported to not have or have a very low expression of key elements of BMP/Smad pathway, such Smad4, ALK6 or phospho-Smad1/5/8, suggesting that this pathway may not be functional (145, 146). The 4 cell lines with the BMP signaling pathway theoretically functional only the AGS and MKN28 presented a negative correlation between the miR-125a-5p and *Dies1* expression, that may be associated with the regulatory function of this miRNA. The miR-125a-5p may regulate the *Dies1* expression in AGS and MKN28 cell lines by a feedback loop between miR-125a, BMP4 signaling pathway and *Dies1* as described by Parisi, et al. (18). However in KATO III cell line, the BMP/Smad signaling pathway appears to be inactive, and therefore miR-125a-5p is likely not be regulated by this pathway. These results represent an association in cell lines and further studies are need to confirm this as regulatory mechanism for *Dies1* expression in this setting.

In summary of this part of the work, DNA methylation can be associated with *Dies1* gene down-regulation in AGS and GP202 cell lines and miR-125a-5p may also be a regulatory mechanism for KATO III, AGS and MKN28 cell lines. However the non-existence of

mutations lead us to exclude this genetic alteration as one of the possible mechanisms for *Dies1* down-regulation. Also the mechanisms that lead to the low mRNA levels of *Dies1* in MKN45, IPA220 and GP202 remain unclear. However others genetics and epigenetics mechanisms can explain the regulation of mRNA levels such: deletion of chromosomal regions bearing the *Dies1* gene (loss of heterozygosity). The presence of heterozygosity in the SNP alteration in KATO III, NCI N87, GP202 and MKN28 cell lines reveal the presence of the 2 alleles in these cell lines, however in MKN45 and IPA220 the homozygous state at *Dies1* SNPs does not allow us to evaluate the presence of the 2 alleles. Alternative splicing is a post-transcriptional mechanism with a significant role in cancer and can also lead to no detection of the transcript of interest (147). *Dies1* presents 3 possible transcripts, 2 of them were not detected by the probe used in qRT-PCT, therefore alternative splicing may lead to a down-regulation of the *Dies1* transcript. Other mechanisms can also induce a gene down-regulation, such as modification of nucleosomal histones, repression of transcription by repressor transcription factors or even regulation by another miRNA.

The evaluation of *Dies1* expression in our gastric cancer series revealed a different expression of this gene compared with the previous tumour cases on preliminary results. In our series *Dies1* displayed an increase expression in tumour samples compared with the normal pair, also, this difference was present in the intestinal type but not in diffuse histotype.

These results were not consistent with the previous series and the cell line results. In addiction to the fact that were 2 distinct series of gastric cancer, we used normal-tumour paired samples that allow us to eliminate the inter-personal variability, whereas the previous series used a pool of normal samples, not paired with the tumour samples. These difference can influence and lead to a different result between the 2 series. Besides this difference, we cannot exclude the fact that in some cases we used the normal adjacent mucosa as normal sample that, besides its had a non-tumorous morphology, it can have already some alterations involved in carcinogenesis that can dilute the differences between the normal and tumour sample.

Gastric cancer is a heterogeneous tumour that frequently present on tumour microenvironment (TME) a high inflammatory cell infiltration. Some studies reported that *Dies1* (termed VISTA) was highly expressed on myeloid cells, such macrophages, monocytes and dendritic cells, and expressed at lower levels in $CD4^+$ and $CD8^+$ T-cells (2, 31, 148). Recent reports disclose the function of *Dies1* in cell immunity: this gene expressed on antigen-presenting cells (APCs) directly supress $CD4^+$ and $CD8^+$ T-cell

proliferation, cytokine production, T-cell activation and T-cell-mediated immune responses (2, 31, 149). Le Mercier and colleagues found a high expression of Dies1 protein in myeloid cells population on TME in murine cancer models, such bladder and melanoma tumour models (31, 149). Also in this study the authors reported that the treatment with a mAb against Dies1 in melanoma murine cancer model and in the transplantable tumour model alter the suppressive cellular signature of the TME, by reducing the tumour-infiltrating monocytic myeloid-derived suppressor cells while increasing the frequency of infiltrating effector T cells. This was accompanied by the enhanced proliferation, activation and effector function of tumour-infiltrating T cells, which likely contribute to impair tumour growth (31, 149). Also the effects of Dies1 mAb treatment facilitate the establishment of an immune-stimulatory TME, which lead to enhance antitumor immunity (31, 149).

Taking these studies in consideration, we cannot exclude the possibility that the over-expression of *Dies1* gene in some tumour samples in our gastric cancer series can be due the expression of this gene in the inflammatory cells. To exclude this cell "contamination" and study only the tumour cells in further studies, we must do a micro-dissection of tumour cells, or perform a protein characterization that can unclear the source of *Dies1* expression.

In present study we already tried to perform the protein staining for better characterize the role of Dies1 in gastric cancer. However the antibody instability, and the heterogeneous staining did not permit us to characterized the Dies1 protein in normal mucosa and in gastric cancer samples. The protein characterization in mouse tissue seems to have better results, this could be due the controlled technical procedure that permitted improved the antigen epitope conservation (150). Besides the controlled histological technique the antibody maintained an instable staining with different intensity in the same tissue and using the same protocol. This seems also be a recurrent problem, once the studies that performed a Dies1 protein characterization had to use an epitope tagging technique or generated their own antibodies, reflecting the lack of specific antibodies commercially available (1, 2, 5). In order to study this protein in the tissue context, we must produce our own antibody or overexpress the protein bound to a tag.

The results of a preliminary study performed in our group revealed that *Dies1* expression varies along the EMT/MET process in an *in vitro* model, and seemed to follow the epithelial markers. This result prompted us to study the association of *Dies1* expression in gastric cancer with an epithelial and mesenchymal marker, which are often associated with the EMT process. The epithelial marker chosen for us to perform this study was the E-cadherin (*CDH1*), that besides being a classical marker in EMT process in many *in vitro*

studies, the alteration of this protein in gastric cancer is associated with loss of cell differentiation, tumour development and invasion (111)

N-cadherin (*CDH2*) is a protein often associated with EMT process and is known as a mesenchymal cadherin. In epithelial cancers N-cadherin is frequently studied as a mesenchymal marker and in some gastric cancer studies has been associated with unfavourable prognosis, and suggest playing a role as a pro-migratory cadherin (123, 151). These 2 cadherins are often described in literature to have a negative correlation and to be associated with cell motility and tumour invasion. The inverse correlation of *CDH1* and *CDH2* was also present in the *mesenchymal type* of the molecular gastric cancer classification (85).

In our gastric cancer series the overall expression of *CDH2* present a similar expression between normal and tumour samples, also there was no difference in *CDH2* expression in the different histological type. However, when we analysed the gene expression normalized for the normal counterpart we can verify that from 7 cases with an overexpression of *CDH2*, 5 of them belongs to the diffuse type. The association of increased N-cadherin (*CDH2*) expression and the diffuse histotype was also obtained in previous studies (85, 123, 152). Besides these results, there was no relation with the increased expression of *CDH2* and a decreased expression of *CDH1* in our gastric cancer series, what excludes the presence of a negative relation between these 2 markers at mRNA level in our gastric cancer series.

The analyses of protein expression could give us a better relation between the N-cadherin and E-cadherin expression, however the staining of N-cadherin in our gastric cancer series was negative in all cases, including the cases with an overexpression at mRNA level. This fact was also observed in another study that compared the mRNA expression and the N-cadherin protein levels, and only one case resulted in immunodetection (152). Posttranslactional modifications or other inactivation mechanism, such proteolytic cleavage, can create alterations in protein conformation and impossibility the antibody binding. These alterations must be limited to the tumour cells, since the positive internal control (ganglion cells) had a specific positive staining. Also the low levels of N-cadherin protein in tumour cells may not be sufficient and be below the sensitivity limits of antibody detection or even the mRNA may not be translated into protein, which can explain the lack of detection of this protein.

Our results also lead us to conclude that N-cadherin levels do not vary significantly in our gastric cancer series. Others markers, as example of vimentin, fibronectin, S100A4, phosphor-SRC and Snail1 must be further studied to better characterize our tumour series

about the presence of mesenchymal characteristics associated with EMT process and correlate with *Dies1* expression.

Contrarily to *CDH2*, *Dies1* expression seems to have the same trend than the epithelial marker, *CDH1*. Both genes had an increase expression between normal and tumour samples, this increase was also significative in intestinal type but not in diffuse type. In the analyses of the expression of this 2 genes when normalized for the normal pair, 74% (14/19) of the cases had a concordant expression of *CDH1* and *Dies1* (the 2 genes had a higher, lower or the same expression compared to the normal pair).

All these results allowed us to demonstrate that *Dies1* and *CDH1* expression seem to have an association in our gastric cancer series. Similar to preliminary results of EMT/MET model, in gastric cancer *Dies1* seems to accompany the epithelial marker *CDH1*. However this results concerns only mRNA levels and *Dies1* expression was only associated to one epithelial marker that given us a lack of information about its association to epithelial characteristics.

The detection of E-cadherin protein provided us with better information about the presence and the correct function of this protein, once the mRNA levels did not correspond to protein levels. E-cadherin may suffer post-transcriptional alterations that lead to a loss of protein expression or loss of normal pattern, and therefore affect the correct function of the protein. This is clear in our gastric cancer series for example, 4 cases with an increased expression of *CDH1* at mRNA level do not have expression of E-cadherin protein. Other cases had an increased expression of *CDH1* compared to the normal pair and had an abnormal protein expression (incomplete staining at membrane, cytoplasmic staining, dotted pattern). These results reflect the importance of the protein analyses to characterize the role of Dies1 in gastric cancer and its association with the epithelial or mesenchymal marker. Further studies need to be performed to associate E-cadherin and Dies1 at protein level, and associate Dies1 with others epithelial markers.

The following scheme represents a resume of the present study (**figure 21**).

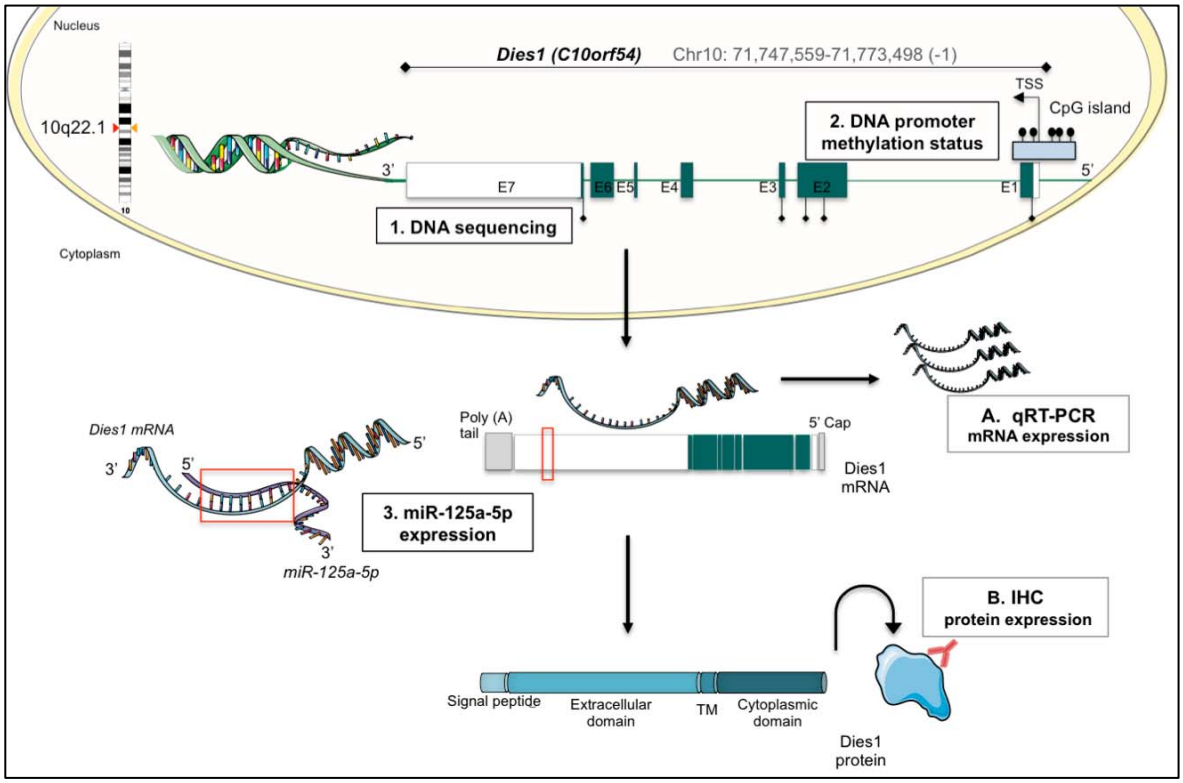


Figure 21 – Schematic representation of Dies1 study. A. Quantification of *Dies1* mRNA levels using qRT-PCR; **B.** IHC techniques used for characterize Dies1 protein, however due technical problems this objective was not successful accomplished. **1.**, **2.** and **3.** Different regulatory mechanisms for *Dies1* expression studied in present work. Black lines in DNA sequence represent the alterations found in gene sequence. E, exon; TSS, transcription start site; TM, transmembrane domain.

CHAPTER 6

CONCLUSION

CONCLUSION

In present work we studied the expression of *Dies1*, a gene involved in ESC differentiation, and the possible regulatory mechanisms of this gene in gastric cancer.
This gene had a lower expression in almost all gastric cancer cell lines studied compared with the normal counterpart, this results was consistent with the previous one achieved in a series of gastric cancer and allows us to study a possible regulatory mechanism that lead to a down-regulation of *Dies1* expression.
The analysis of 3 possible regulatory mechanisms, in particular, gene sequence alterations, promoter methylation and regulation by miR-125a-5p, lead us to suggest that the last 2 mechanism seem to regulate *Dies1* gene expression. Promoter methylation was the possible mechanism controlling the *Dies1* expression in 2/7 gastric cancer cell lines but it was not found in primary gastric cancer samples with a down-regulation of *Dies1* mRNA. Therefore *Dies1* promoter methylation is likely a rare regulatory mechanism for *Dies1* expression in gastric cancer. The expression regulation by miR-125a-5p seems to be present in 3/7 cell lines, but this mechanism was not explored in primary tumour samples. Overall, we believe that others mechanism may be involved in *Dies1* gene expression regulation in gastric cancer.
The second aim of this study revealed that in our gastric cancer series, *Dies1* expression had an apparent association with the expression of the epithelial marker, *CDH1*, which may indicate *Dies1* as a gene associated with an epithelial differentiation.

Our study was a first reporting in analysis on the role of *Dies1* in gastric cancer, and suggests that *Dies1* is differentially expressed in gastric cancer and that different mechanisms may explain its expression modulation, namely promoter methylation and expression of miR-125a-5p. The correlation observed between *Dies1* and *CDH1* expression suggests an association of *Dies1* with epithelial differentiation.

CHAPTER 7

FUTURE PERSPECTIVES

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The work development in this thesis was the first approach for study the role of *Dies1* in gastric cancer. Many issues and unanswered questions still need to be clarified and future works are necessary to better understand the role of this gene in gastric cancer such:

- Expression analysis of *Dies1* in a larger gastric cancer series.

- Microdissection of tumour cells and analysis the *Dies1* expression to exclude the possibility that the increased of its expression was due to a "contamination" by the inflammatory infiltrate in TME. To exclude this possibility and to characterize the expression of Dies1 protein in normal gastric mucosa and in gastric cancer an IHC technique with an optimized antibody must be accomplish.

- Characterize the expression of Dies1 protein concomitantly with the EMT markers, such vimentin, fibronectin, phospho-Src, Snail, Twist, and others to study the association of this protein expression with the EMT process in gastric cancer.

- Study the association of Dies1 mRNA or protein expression with the BMP signalling pathway. In ESC differentiation Dies1 function as a co-receptor of BMP signalling pathway, and its down-regulation lead to a decrease of this signalling function, also the regulation of Dies1 by miR-125a-5p seems to be related with this signalling pathway. In gastric cancer Dies1 can also be involved in BMP pathway, so, to clarify this association, and study the association with miR-125a-5p regulation and BMP pathway further studies must be done.

- Perform further studies to prove that the hypermethylation and miR-125a-5p are regulatory mechanisms of *Dies1* expression in gastric cancer. Examples of studies that can be performed are: treatment with 5-aza-2-deoxycytidine to prove that promoter methylation is a regulatory mechanism; analysis of the expression of miR-125a-5p in the gastric cancer series; blockade of the miR-125a-5p and measurement of the expression of *Dies1*.

Further studies are necessary to clarify the unanswered questions raised with the present study.

CHAPTER 8

REFERENCES

REFERENCES

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