GENETIC MARKERS OF HPV INFECTION AND CERVICAL CANCER: CLINICAL UTILITY

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ABBREVIATIONS LIST

A

ANOVA- Analysis of Variance

C

CIN- Cervical Intraepithelial Neoplasia

CIS- Carcinoma *in situ*

Ct- Cycle threshold

D

DNA - deoxyribonucleic acid

dNTP- deoxyribonucleotide

F

FDA- Food and drug administration

H

HC2- Hybrid Capture 2

HPV- Human Papillomavirus

HR-HPV- High Risk Human Papillomavirus

HSIL- High-grade Squamous Intraepithelial Lesion

I

ICC – Invasive Cervical Carcinoma

L

LSIL- Low-grade Squamous Intraepithelial Lesion

LR-HPV- Low-Risk Human Papillomavirus

M

mRNA- Messenger RNA

miR- Mature microRNA

miRNAs- microRNAs

P

PCR - polymerase chain reaction

Pre-microRNA- precussor microRNA

Pri-microRNA- primary microRNA

R

Real-Time PCR – *Real-Time Polymerase Chain Reaction*

RFLP – *Restriction Fragment Lenght Polymorphism*

RNA-Ácido ribonucleico

ROC- Receiver operating characteristic

S

SPSS- Statistical Package for Social Sciences

Q

qPCR- Quantitative Polymerase Chain Reaction

V

VC- Variance coeficient

OUTLINE OF THE THESIS

OUTLINE OF THE THESIS

In **Chapter I,** the rational for this study will be presented focusing the need for identification of predictive/prognostic biomarkers of persistent HPV infection. Additionally, the aims of this thesis will also be described in this chapter.

In **Chapter II**, it will be presented a review of literature which will briefly explain the potential impact of microRNAs as biomarkers of HPV-associated carcinogenesis, with a major focus on the potential of miR-125b and miR-34a. This chapter is presented as the following article:

Ribeiro J, Sousa H. ROLE OF MICRORNAS IN CERVICAL CANCER: ARE MIR-125B AND MIR-34A GOOD ENOUGH? *Cancer Letters* (submitted)

In **Chapter III**, it will be characterized the profile of HPV-genotypes in women from the northern region of Portugal with major focus on the characterization of HPV16 infection and HPV16 physical status. This chapter is presented as the following article:

Ribeiro J, Teixeira D, Dias J, Monteiro P, Loureiro J, Baldaque I, Medeiros R, Sousa H. MOLECULAR CHARACTERIZATION OF HPV GENOTYPES IN PORTUGUESE WOMEN AND CONTRIBUTES FOR FUTURE APPROACHES. *Journal of Medical Virology* (submitted).

In **Chapter IV**, it will be shown the results of miR-125b and miR-34a expression in different cervical lesions, including carcinomas, discussing its utility as predictive/prognostic biomarkers of HPV infection and cervical cancer development. This chapter is presented as the following article:

Ribeiro J, Dias J, Monteiro P, Loureiro J, Baldaque I, Medeiros R, Sousa H. miR-34a AND miR-125b EXPRESSION IN HPV INFECTION AND CERVICAL CANCER DEVELOPMENT. *BMC Cancer* (submitted).

In **Chapter V,** it will be summarized the importance of the results obtained in this thesis. Up to our knowledge, this is the first study to evaluate the expression of miR-34a and miR-125b in cervical tissues as predicte/prognostic markers of HPV infection outcome. Additionally, it will be suggested future directions for research in this field of knowledge.

Finally, in **Chapter VI,** it will be presented the references of the thesis.

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ABSTRACT

ABSTRACT

Cervical cancer constitutes a serious public health issue representing the third most commonly diagnosed cancer in females with approximately 530 000 of new cancer cases each year and a total of 275 100 deaths among females in 2008. In Portugal cervical cancer is the 4th most frequent cancer in with 950 new diagnosis and 378 deaths per year.

Persistent infection with one or more carcinogenic types of *human papillomavirus* (HPV) has been established as the main etiological factor of squamous intraepithelial lesions of the cervix which may progress to high-grade dysplasia or to invasive carcinoma. The majority of HPV infections are asymptomatic and the outcome of HPV infection is variable. Despite recent advances, clinicians still demand for the identification of useful biomarkers of prognosis of HPV infection and progression of lesions.

This study was performed with exfoliated cervical cells collected from women with different cervical lesions and cancer. Firstly, we aimed to characterize HPV genotypes in our population. Our results showed that HPV16 is the most frequent types among type followed by HPV31, 58, 39, 53 and 66. Surprising, HPV18 proved to be a rare genotype in our population. Physical status of HPV's genome has been described as potential prognostic marker and therefore we determined the viral physical status in HPV16 positive cases. Results showed that it was possible to detect integrated forms in early lesions of the cervix.

Another aim consist in to analyze the expression of two miRNAs (miR-125b and miR-34a) in cervical cancer tissues and to evaluate its impact as predictive/prognostic biomarkers of lesions and cancer of the cervix.

Regarding results obtained to miR125b expression, we observed a two-fold increased levels of miR-125b among normal cases with HPV infection. Moreover, data showed a gradually trend to down-regulation of miR-25b expression with lesions progression, becoming a significant decreased expression in women with carcinoma. In contrast to previous reports, miR-34a expression was found increased among women with normal cervix and HPV infection however, when comparing LSIL, HSIL or CIS/ICC with reference group we observe no significant differences in relative expression. This study revealed that levels of miR-34a remain constant among different cervical lesions and cancer tissues and results regarding miR-125b expression suggest that miR-125b could probably be used as useful tool for cervical lesions diagnosis/prognosis.

RESUMO

RESUMO

O cancro do colo do útero assume-se como um sério problema de saúde pública, representando a terceira neoplasia ginecológica mais frequente em todo o mundo com cerca de 530 000 casos diagnosticados por ano. Em Portugal, o são registados cerca de 950 novos casos e 378 mortes por ano tornando-se a 4ª neoplasia mais frequente no país.

A infecção persistente com um ou mais tipos de *Human papillomavirus* (HPV) de alto risco foi estabelecida como o principal fator etiológico de lesões intra-epiteliais do colo do útero que podem evoluir para lesões de alto grau ou carcinoma invasivo. A maioria das infecções por HPV são assintomáticas, contudo o resultado da infeção por HPV é variável. Apesar dos avanços recentes, os clínicos exigem cada vez mais a necessidade de identificar biomarcadores uteis na definição do prognóstico da infeção por HPV.

Este estudo foi realizado usando raspagens do colo do útero de mulher com diferentes lesões, incluindo carcinomas do colo do útero. O primeiro objetivo foi caracterizar a infeção por HPV na população em estudo. Os resultados mostram que o HPV16 é o genótipo mais frequente seguido pelo HPV31, 58, 39, 53 e 66. Notavelmente, o HPV18 mostrou ser um genótipo raro entre a nossa população. O estado físico do genoma viral tem sido descrito como um potencial biomarcador de prognóstico, nesse sentido, foi verificado o estado de integração dos casos HPV16 positivos. Os resultados obtidos mostraram que é possível encontrar formas integradas em lesões de baixo grau, destacando o facto que a integração viral pode ser um evento precoce no processo de carcinogenese. No entanto, mais estudos

Outro objetivo consistia em estudar os níveis de expressão de dois miRNAs (miR-125b e miR-34a) em células do colo do útero e avaliar o seu potencial como biomarcadores de lesões e cancro. Considerando os resultados de expressão do miR-125b, obteve-se um aumento significativo da expressão em raspagens com citologia normal HPV positivas. Além disso, os resultados mostraram uma tendência para a diminuição gradual da expressão do miR-125b ao logo da progressão das lesões. Nos carcinomas a expressão do miR-125b encontra-se substancialmente diminuída. Em contraste com informação descrita previamente, o miR-34a encontra-se aumentado nos casos normais HPV positivos, no entanto, nos casos com lesões de baixo, alto grau e carcinomas não se verificaram diferenças estatisticamente significativas nos níveis do miR-34a. Em suma, os nossos dados revelaram que a expressão do miR-34a mantem-se contante entre as diferentes lesões/cancro do colo uterino, enquanto que expressão do miR-125b encontra-se alterada sugerindo a possibilidade do miR-125b ser um bom marcador preditivo para lesões do colo do útero e infeção por HPV.

CHAPTER I

BACKGROUND

Cancer is a major health problem worldwide, being one of the major death causes even in developed countries. Currently, cervical cancer is the third most common cancer among women and it represents a severe threat to women´s live. Data from World Health Organization (WHO) showed that there were 590 000 new cases of cervical cancer and over 275 000 deaths in 2008 with huge differences in incidence rates between developed and developing countries. [Frazer, 2004; Sankaranarayanan and Ferlay, 2006; Jemal et al., 2011]. Interestingly, the incidence of cervical cancer has been changing in developed countries: while in early 50's the incidence started to increase due to the appearance of better diagnostic tools, in early 90's it has started to decrease due a better clinical approach and better use of preventive measures.

Nowadays, it is established that the persistent infection by one or more carcinogenic types of Human Papillomavirus (HPV) is the main etiological factor for cervical cancer development. [zur Hausen, 1991; zur Hausen, 2009a; Zur Hausen, 2009b; Zur Hausen and Rosl, 1994]. The procedures for cervical cancer detection and screening are based in screening programs with cytology (Pap smear test) and more recently with the detection of HPV DNA. The establishment of coordinated screening programs has reduced substantially rates of incidence and mortality of cervical cancer, however, clinicians still demand for the identification of useful predictive/prognostic biomarkers for HPV infection and progression of lesions.

The majority of HPV infections are asymptomatic and are efficiently controlled by the immune system, therefore, the outcome of HPV infection is variable and the infection is usually transient and complete resolution is generally common within 6 to 12 months [Ho et al, 1998; Woodman et al, 2007]. However, persistent infection with one or more carcinogenic types of HPV may lead to the development of intraepithelial lesions, which may progress to high-grade dysplasia or in severe cases to invasive carcinoma [Woodman et al, 2007].

In the past decade there have been several studies that attempt to develop proof-valuable biomarkers for HPV infection outcome based on the distinct events of HPV-related carcinogenesis and cellular modifications caused by HPV infection (Hwang et al, 2012). Recent data point to the importance of microRNAs (miRNAs) in different cancers. These single-stranded RNAs sequences (~22 nucleotides) participate in several cellular processes, regulating up to 30% of human protein-coding genes, by suppressing RNA translation or inducing messenger RNA (mRNA) degradation. Hence, miRNAs are thought to represent a new approach in the diagnosis and prognosis of cervical cancer [Melo and Esteller, 2011; Pereira *et al*, 2010]. Moreover, the identification of different tumor-specific microRNA signatures could be very helpful to distinguish pre-malignant lesions from invasive cancers. In fact, studies have been described several interactions between microRNAS and HPV oncoproteins and recent data have been demonstrated that several miRNAs are deregulated and presents abnormal expression in lesions and cancer tissues of the cervix [Wang et al, 2008; Pereira et al, 2010, 2007; Martinez et al, 2008].

The main aim of this thesis is to characterize the expression of two miRNAs (miR-125b and miR-34a) in different cervical lesions including cancer. Both miR-125b and miR-34a have been described as interacting in crucial steps of cancer development. By characterizing the expression of miR34 and miR125b in cervical lesions we expect to be able to associate levels of expression with different outcomes of HPV infection and cervical lesions; and therefore to determine the impact of these miRNAs as predictive/prognostic biomarkers.

CHAPTER II

ROLE OF MICRORNAS IN CERVICAL CANCER: ARE MIR-125B AND MIR34A GOOD BIOMARKERS?

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Abstract

MicroRNAs are non-coding RNAs with important functions in several biological processes, such as, regulation of cell cycle, immune response, inflammation, and apoptosis. In fact, deregulation and abnormal expression of these molecules is associated with human pathologies including cancer and several have already emerged as potential prognostic biomarkers in different neoplasias. miR-34a is directly regulated by p53 and acts as tumor suppressor while miR-125b plays a significant role in immune response and apoptosis. In cervical carcinogenesis, HPV proteins seem to interact with both miR-34a and miR-125b changing its expression and promoting persistent infection and cervical cancer development. In this review we describe the potential role of miR-125b and miR-34a in cervical carcinogenesis, including interaction with HPV and mechanism of deregulation. Additionally, their clinical applications in cervical cancer as prognostic/predictive biomarkers are also briefly discussed.

Keys words:

Cervical cancer, HPV infection, miR-34a, miR-125b, biomarkers

Cervical Cancer and HPV carcinogenesis

Worldwide, cervical cancer is the third most commonly diagnosed cancer in females with approximately 530 000 of new cancer cases each year and a total of 275 100 deaths among females in 2008 [1]. However incidence rates show to be different between developed or developing countries. Recent data reveals that cervical cancer is extremely common in countries such as Central and South America, Eastern Africa and South-East Asia in contrast to developed counties [2; 3]. Over the past 100 years, the incidence of cervical cancer has been changing a lot in developed countries. While in early 50's the incidence started to increase due to the existence of better diagnostic tools, in early 90's it has started to decrease due a better clinical approach and better use of prevention measures.

The use of Pap smear test in screening programs has proved to be useful for the prevention of cervical cancer in the last 30 years. Although histological examination of colposcopy-guided biopsies is still considered the confirmatory exam in the assessment of cervical lesions, screening with cytological examination is the most used worldwide. This technique is limited to the interpretation of the morphological alterations of tissues and do not provide information regarding the infection and the risk of progression [4].

Since early 90's that scientific community understands better the carcinogenic model of cervical cancer. There were many studies until the clear observation that persistent infection with one or more carcinogenic types of *human papillomavirus* (HPV) was the etiological factor for the development of cervical cancer and that the infection of cervix by HPV is followed by a cascade of events that lead to the development of squamous intraepithelial lesions of the cervix which may progress to high-grade dysplasia or to invasive carcinoma [5; 6].

In the past decade there have been several studies that attempt to develop proof-valuable biomarkers for HPV infection outcome based on the distinct events of HPV-related

carcinogenesis and cellular modifications caused by HPV infection [7]. The greatest hallmark of HPV-associated carcinogenesis is thought to be the integration of the viral genome into the host genome. This event leads to HPV-E2 gene disruption and therefore deregulating both viral and host genome activity. The loss of HPV-E2 results in the constitutive expression of both HPV-E6 and HPV-E7 proteins leading to oncogenic transformation of cells: HPV-E6 binds p53 inducing its degradation by the ubiquitin-proteasome pathway promoting p53-dependent apoptosis activation; and HPV-E7 modifies pRb phosphorylation promoting cell cycle checkpoints escape and cell proliferation [8; 9]. Despite these evidences, the utility of HPV-genome integration into host-cells as a biomarker in clinical decision is doubtful and therefore several other biomarkers have been studied, such as viral load, detection of E6/E7 mRNA and expression of p16 in cervical tissues [7]. Recently, scientific community has started to debate whether miRNAs can be used as possible markers for the occurrence and development of the HPV-associated cancers.

MicroRNAs

Small non-protein-coding RNAs are rising as a new class of regulatory genes with significant relevance in cellular processes. In 1993, small RNAs were first described as essential regulators for post-embryonic development of *Caenorhabditis elegans* [10; 11], and since 2001 designated as "microRNAs" [11; 12; 13]. microRNAs (miRNAs) are small non-coding RNAs of 18-25 nucleotides in length (see miRBase, http://microrna.sanger.ac.uk/), generated from hairpin transcripts, that potentially target up to one-third of human coding genes managing normal cellular processes, including proliferation, differentiation and apoptosis [10; 11; 14; 15; 16; 17]. Additionally, they are described as key regulators in many diseases including, neurological disorders, cardiovascular diseases, viral infections and cancer [18].

MicroRNA genes are thought to be located in the introns or long non-coding RNAs transcripts, as well in independent transcription units with specific promoters and polyadenylation signals [17]. Approximately 50% of mammalian microRNA loci are close to other miRNAs regions and are transcribed as polycistronic messages in single transcript units or are overlapped in the host transcripts, within exons or introns, depending on the splicing processes [17].

Structurally, miRNAs are part of a long transcript known as pri-miRNA (hairpin precursor) that contain a 5´-cap structure, a poly(A)+ tail, a stem–loop structure and also may include introns. In the nucleus, pri-miRNA is processed by *Drosha* complex into pre-miRNA (~70 nucleotides) that are exported to cytoplasm by *exportin-5* and cleaved by the *Dicer* complex to become a mature duplex miRNA [15; 17; 19]. This duplex miRNA, which includes two strand: the miRNA-5p and the complementary strand known as miRNA-3p, is then uncoiled, and while the mature miRNA (also known as guide strand) is loaded into the RNA-induced silencing complex (RISC) the other strand (passenger strand) is usually degraded [15; 19].

MicroRNAs and Cancer

The aberrant expression or alterations of miRNAs are associated with human pathologies, including cardiovascular disorders, inflammatory diseases and cancer [20; 21]. Studies have confirmed that miRNAs are able to regulate the expression of 30-60% of human proteins [11]. These small molecules are thought to regulate gene expression by binding mRNA targets and promoting their degradation and/or translational inhibition, although, some studies suggested that miRNAs can also increase the expression of a target mRNA [17]. Nucleotides 2–7 of the miRNA are nominated by "seed region" and they are responsible for binding specificity to the target mRNA. However, recent data pointed that not only these molecules can target mRNA but also DNA and proteins [17]. Another important fact of these molecules is that each miRNA may target several different mRNAs and a single mRNA can be targeted by several miRNAs [17].

Recent studies have suggested that deregulation of miRNAs caused by both genetic and/or epigenetic mechanisms potentiate cancer development [17]. In fact, genome-wide transcriptional analysis have showed that the profile of miRNA expression seems to be sufficient to classify a variety of human cancer suggesting a diagnostic value for these molecules [22]. Moreover, differences in miRNA expression profiles seem to define specific signatures of various cancers and even allow identifying specific oncogenic abnormalities [11; 14].

Several studies have been addressing on the impact of miRNAs in tumor development and consequently the number of these molecules involved in human cancer has been growing [11]. The biological functions of miRNAs are highly dependent on cellular context, which may be due to differential expression of their target mRNAs. miRNAs are known to act as both regulators and targets of oncogenes and tumor suppressor genes depending on specific tissues genes by modulating the levels of specific proteins with tumor suppressor or

oncogenic potential (Table I) [11; 18]. Further understanding of the expression and function of miRNAs in different cancers is thought to provide in the near future the development of better diagnostic and therapeutical approaches against cancer, mainly by allowing the identification of pre-neoplastic lesions and better follow-up of patients [23].

MicroRNAs and cervical cancer

Recent studies suggest miRNAs as possible markers for the occurrence and development of the HPV-associated cancers and the differences in tumor-specific miRNA signatures might be helpful to distinguish different cervical lesions [24]. Studies have been describing several interactions between miRNAs and HPV oncoproteins [25; 26; 27] and, in fact, specific miRNAs have been located in cancer-related genomic regions, which include fragile sites at or near HPV integration sites [28]. Moreover, recent studies using microarrays methods have characterized the expression of several miRNAs in cervical tissues [24; 29].

Microarrays analysis have demonstrated that miRNAs involved in important cell events, such as miR-21, miR-24, miR-155, and miR-205 are up-regulated, whereas many others are down-regulated (eg. miR-100, miR-let7b, miR-143, miR-145) [29]. Li et al, 2011 have also described that miR-100 is significantly and gradually reduced from pre-neoplastic lesions to invasive cervical cancer [30]. Nevertheless, the author has shown that E7/E6 expression did not change miR-100 levels and therefore, it is still unknown if the downregulation is a cause or consequence [30; 31]. Moreover, *in vitro* experiments have been performed to study interactions between microRNAs and HPV oncoproteins with interesting interactions observed [27; 30; 31; 32].

In this review, we aimed to describe the mechanisms of miR-34a and miR125b in cervical cancer development. Additionally, their potential clinical applications and prospects in cervical cancer, such as biomarkers and clinical therapies, are also briefly discussed.

miR-125b

miR-125 is a highly conserved miRNA common among various species. In humans, miR-125 family is constituted by three homologs (hsa-miR-125a hsa-miR-125b-1 and hsa-miR-125b-2) whose genes are located in different chromosomes (chr19q13, chr11q24 and chr21q21, respectively) (see mirBase: http://microrna.sanger.ac.uk/). Despite different location within the genome, they have the common primary transcript that also contains the miR-99/miR100 and let7 family member. Although it remains unclear if the three different miRNAs produced by the transcript are functionally linked, it is supposed that duplicated clusters are under distinct transcriptional control displaying different expression patterns and functions [33].

The study of miR-125b family expression is still controversial, with some results showing that it depends on the type of tissue, mainly due to the tissue microenvironment. As a result, miR-125b has been described as potentially involved in inflammation, cell proliferation and cell cycle regulation which makes it difficult to fully characterize this molecule in different diseases [20; 34; 35; 36].

miR-125b is thought to act as both oncogene and tumor suppressor: while overexpression of miR-125b is reported in prostate cancer and hematological malignances, where it seems to act as oncogene [37; 38; 39]; down-regulated expression has been described in breast, ovarian, hepatocellular and thyroid carcinomas, therefore assuming a tumour suppressor role [36; 38; 40]. In fact, Zhang and colleagues proved that miR-125b expression in breast cancer cells suppress proliferation and induces G1 cell-cycle arrest [36; 41].

Despite its involvement in different pathways, miR-125b was described as a negative regulator of p53 and therefore regulates the potential p53-dependent transactivation activity, thus leading to increased cellular instability and ultimately decreased apoptosis [35; 41]. The

oncogene/tumor suppressor balance is probably explained by the direct interaction of miR-125b with apoptosis, where it seems to play a major cellular role: either by suppressing the pro-apoptotic molecule Bcl-2 Antagonist Killer 1 (Bak1) (anti-apoptotic role); or by regulating the expression of Mcl-1, Bcl-w and IL-6R (pro-apoptotic role) [42]. Additionally, studies have reported that miR-125b is also able to suppress cell proliferation and promote apoptosis by targeting ERBB2, ERBB3, ETS1 and Bcl-2 [36; 38; 43].

In the past years, miR-125b has also been studied in viral associated diseases due to its possible role in inflammation and immune response regulation [44; 45]. Wang and colleagues, using microarray data, have described that miR-125b is down-expressed in cervical cancer due to its possible interaction with HPV proteins, a theory that Li and colleagues have corroborated [29; 31]. Literature also reveals that there is a strong homology between miR-125b and the HPV-L2 protein from different HPV genotypes, suggesting a possible direct interaction between miR-125b and HPV. Nuovo and colleagues revealed that HPV-L2 induces inactivation of miR-125b in HPV infected cells and that this event seems to be associated with cytological changes of the koilocytes [46]. These data also point to a marked reduction of miR-125b expression in HPV-infected tissues when compared to normal tissues. Up to date, there is no clear explanation about the biological processes that leads to miR-125b down-regulation, nevertheless, it is plausible to presume that this molecule might play some role in HPV-associated cancer development. Moreover, authors are suggesting that it could be used as potential prognostic marker predicting HPV outcome and cervical lesions progression.

miR-34a

The miR-34 family is composed by three miRNAs (miR-34a, miR-34b and miR-34c) encoded by two different genes: miR-34a is encoded by its own transcript located at 1p36; whereas miR-34b/c share a common primary transcript at chromosome 11q23.1 (see mirBase: http://microrna.sanger.ac.uk) [23]. Genome analysis reported that both genes have a proximal promoter region that contains a p53 binding site, thus members of the miR-34 family are described as direct p53 targets and by targeting miR-34 family, p53 is able to induce apoptosis, cell cycle arrest and senescence [23; 25; 26; 47; 48; 49]). In fact, several studies have shown that miR-34a seems to be the most significantly induced miRNA after activation of p53 and therefore p53 can regulate the expression of several proteins, even after their mRNAs have already been translated [Bommer et al., 2007; Chang et al., 2007; He et al., 2007a; He et al., 2007b]. In the presence of cellular stress, this type of regulation becomes more advantageous because it does not need the translation of additional effector proteins, which might take long time to act [23].

Despite the majority of studies have implicated the miR-34 family in the p53-network, induced by p53-dependent DNA damage pathway, the consequences of miR-34 activation may vary depending on cellular context [He et al., 2007a; He et al., 2007b]. Bioinformatics systems have been used to predict miR-34 targets providing a better knowledge of its wide-spectrum. Moreover, some of these targets were already validated by experimental analysis and the different roles may depend on the spectrum of miR-34 targets expressed in a specific cell type [He et al., 2007a; He et al., 2007b]. Bommer *et al* showed that miR-34a is expressed at higher levels than miR- 34b/c with the exception of the lung, where these two miRNAs are presumed to have specific functions [23; 47].

Considering that miR-34 family is induced by p53, several studies have been preformed to evaluate the role of miR-34s, especially miR-34a, in different cellular processes in different

neoplasias. Taking into account the role of miR-34 in cell-cycle arrest, senescence and apoptosis as tumor suppressor gene, its permanent inactivation, may be a selective advantage for cancer cells [23]. Decreased expression of miR-34s can be brought to epigenetic mechanisms and mutations of miR-34-encoding genes as well as to mutations and viral inhibitors of p53 [23]. In fact, miR-34 has been reported as donwn-regulated and/or inactivated in several types of cancer, including lung, ovarian, colorectal, pancreatic, urothelial, and renal cell carcinomas [50; 51; 52].

miR-34 family has also been studied for its role in cervical cancer development based on the possible interaction with HPV proteins [53; 54] Recently there were described evidences that expression of miR-34a is significantly lower in High-Risk HPV (HR-HPV) infected tissues [Li et al., 2010]. These evidences associate reduced miR-34a expression with HR-HPV infection and cervical cancer development. In cervical carcinogenesis, HPV-E6 oncoprotein binds to p53 targeting its degradation trough the ubiquitin-dependent proteasome system [9]. The inactivation of p53 leads to a diminished amount of protein available to bind miR-34 p53 binding site promoter region, thus leading to a down-regulation of miR-34a expression in most HPV infected tissues. This theory is supported by some studies that associate viral E6-mediated reduction of p53 and miR-34a family contributes to cell proliferation and transformation [23; 54].

Actually, further studies are need for a better clarification of the biological processes by which miR-34 is down-regulated in cervical carcinogenesis. Moreover, studies evaluating miR-34 levels in cervical lesions and cancer might be important for prediction of HPV infected patients outcome.

Final conclusions

In this article we summarized the current knowledge about the potential role of miR-34a and miR-125b in cervical cancer development. Both miR-34a and miR-125b seems to present

abnormal expression levels in cervical cancer. These two molecules interact with distinct targets and therefore play different functions in important processes, such as, inflammation, immune response, cell cycle, apoptosis and proliferation.

miR-34a is a downstream target of p53-network with key functions in the inactivation of several proteins involved in apoptosis, G_1-arrest, DNA repair and senescence. This regulation is essential for the maintenance of cellular stability and therefore in normal cases, in the presence of cellular stress the activation of p53-network will allow cells to avoid the accumulation of errors and ultimately the development of neoplastic cells. After HPV infection, the activation of p53 is crucial for the control of cell proliferation. Now, we clearly understand that HPV is highly effective in the abolishment of efficient immune response to infected cells, mainly by promoting HPV-genome integration into host cells. The integration of HPV-genome leads to the expression of HPV-E6 protein that targets p53 to degradation trough the ubiquitin-proteasome pathway. Thus, with a decreased availability of p53, the amount of miR-34a will be decreased and therefore its ability to inactivate some proteins involved in apoptosis, G_1-arrest, DNA repair and senescence will allow cells to proliferate and therefore contribute for the development of a neoplastic lesion (Figure 1).

Literature data reveals that miR-125b is a negative regulator of p53 and therefore acts upstream p53 activation. Increased levels of miR-125b are thought to abolish p53 functions by targeting its mRNA and therefore inducing a decreased activation of p53-dependent apoptosis and increasing cellular instability by avoiding G_1-arrest and DNA repair. Moreover, there are some data that refer miR-125b as having an important role in immune response and inflammation. After HPV infection it is expected that HPV would replicate and infected more cells. This process is dependent on effective production of HPV-virions and studies have confirmed that miR-125b has strong homology for HPV-L2, which is crucial for the viral capsid assembly. These data suggest that miR-125b may probably be involved in two distinct

pathways of HPV-associated carcinogenesis. On one hand it is expected that after early infection it will avoid HPV replication and therefore could improve viral clearance. Nevertheless, mirR-125b will also lead to p53-pathway inactivation and therefore cells will be maintained viable with viral genomes inside. This may lead to the increase risk of HPV-genome integration and therefore to increased risk of neoplastic lesion development (Figure 1).

Considering all these data, it is possible that while miR-125b may assume an important role on the initial steps of cervical carcinogenesis, including inflammation and immune response against HPV infection, miR-34a is probably more important in the development of high-grade cervical intraepithelial lesions. This is a theoretical model that still requires confirmation by expression or *in vitro* studies to confirm if miR-34a and/or miR-125b can be used as predictive/prognostic markers in the different steps of cervical carcinogenesis.

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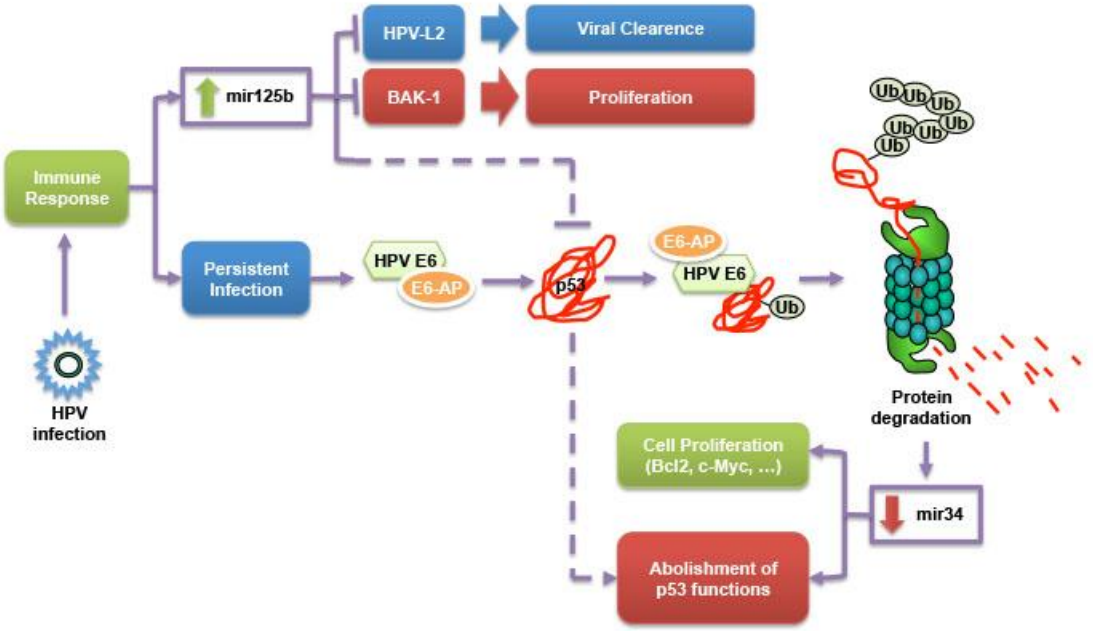
Table I – Human miRNAs targets and functions in carcinogenesis

Human miRNA	Targets	Activity	Reference
Let-7 family	CDC25A CDK6 c-myc RAS	Tumor suppressor	[55] [56]
miR-9	E-cadherin	Tumor suppressor	[57]
miR-15-16 cluster	Bcl2	Oncogene	[58] [59]
miR-18	ATM	Oncogene	[60]
miR-21	hMSH2 CDC25A Bcl2	Oncogene	[61] [62] [63]
miR-34a	Bcl2 Survivin CDK4 CDK6 Cyclin D1 Cyclin E2 c-Myc E2F3 C-Met SIRT1 MTA2 WNT1 LEF1	Tumor suppressor	[25] [23]
miR-124a	CDK6	Tumor suppressor	[64]
miR-125b	p53 Bak-1	Oncogene	[42]
	ERBB2 ERBB3 ETS1 Bcl-2 Mcl-1 Bcl-w IL-6R	Tumor suppressor	[43] [36] [38]
miR-137	CDK6	Tumor suppressor	[64] [65]
miR-146b	NF-kappaB	Tumor suppressor	[66]
miR-205	E2F1 LAMC1	Tumor suppressor	[67]
miR-221	p27 p57	Oncogene	[68]
miR-421	ATM	Oncogene	[69]
miR-504	p53	Oncogene	[70]

Figure 1 - miR-34a and miR-125b interactions and association with HPV



CHAPTER III

MOLECULAR CHARACTERIZATION OF HPV GENOTYPES IN PORTUGUESE WOMEN AND CONTRIBUTES FOR FUTURE APPROACHES

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ABSTRACT

Background: Cervical cancer development is associated with persistent infection by high-risk types of human papillomavirus (HPV) and its detection and genotyping is considered to be crucial to identify women with increased risk of cervical cancer development. Different prevalence of HPVs has been reported in distinct populations and therefore its distribution may determine future clinical approaches and improve the intervention on vaccination programs.

Material and Methods: This study was performed with cervical samples from 95 women with histological confirmation of cervical lesions. HPV was detected by Hybrid Capture 2 (HC2) and genotyping was performed by polymerase chain reaction and restriction fragment length polymorphism (PCR-RFLP) using a protocol previously described.

Results: HPV infection was detected in 42.9% (18/42) of women without lesions, 63.6% (14/22) of cervical intraepithelial neoplasia grade 1, 85.7% (6/7) of cervical intraepithelial neoplasia grade 2, 100% (12/12) of cervical intraepithelial neoplasia grade 3, and 100% (8/8) of cervical carcinomas. Statistical significant differences were found when performing age-stratification of women according to age of 45 years old (p=0.021; OR=2.78). Overall 16

distinct HPV genotypes were detected: 9 high-risk (HPV16, 18, 31, 33, 39, 52, 56, 58 and 59), 2 probable high-risk (HPV53, 66, 82) and 3 low-risk (HPV11, 61, 70). The most prevalent type was HPV16 (40.8%) followed by HPV31 (12.2%), HPV58 (10.2%), HPV39 (6.1%) and HPV53 and 66 (3.8%, each).

Conclusion: This study provides confirms that HPV16 and HPV31 followed by HPV58, 39, 53 and 66 are the most frequent HPV genotypes and that HPV18 is a rare genotype in our population. This information should be included in future approaches, mainly in the decision of future vaccination programs in Portugal.

Keys words:

Human Papillomavirus genotypes, integration, cervical cancer

INTRODUCTION

Worldwide, cervical cancer is the third most commonly diagnosed cancer in females with approximately 530 000 new cancer cases and 275 100 deaths each year. In Portugal, cervical cancer is the 4th most frequent cancer with 950 new diagnosis and 378 deaths per year [Jemal et al., 2011; Pinheiro et al., 2003].

Since early 1990´s that persistent infection by high-risk HPVs is recognized a necessary but non-sufficient condition for the development of cervical cancer [zur Hausen, 1991; zur Hausen, 2009a; Zur Hausen, 2009b; Zur Hausen and Rosl, 1994]. Human papillomavirus (HPV) infection is highly prevalent in sexually active women. Despite majority of HPV infections spontaneously regress in a small percentage of cases the infection persists leading to development of low-grade intraepithelial lesions (LSIL) that might progress to high-grade intraepithelial lesions (HSIL) and, ultimately, develop invasive cervical carcinoma [Woodman et al., 2007].

Pap smear test is actually the most commonly used screening procedure throughout the world for the diagnosis of cervical lesions. However, the detection of HPV DNA has been described as a sensitive test and therefore it has been implemented in some countries [Kuhn et al., 2000; Salmeron et al., 2003; Villa, 2006]. Recently, with the development of genotyping techniques, HPV genotyping allows to indentify women with persistent infection and consequently with increased risk to malignance progression. Integration of HPV genome into the host´s genome is considered the hallmark of HPV-associated carcinogenesis and has been suggested as another important tool to select women with increased risk of cervical cancer, however, the significance of HPV physical status detection remains unclear [Janicek and Averette, 2001; Pett and Coleman, 2007; Ramanakumar et al., 2010].

HPV types have been divided into four groups based on their oncogenic activity: high-risk (HPV 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, and 59), probable high-risk (26, 53, 66, 68,

73, and 82), low-risk (6, 11, 13, 40, 42, 43, 44, 54, 61, 70, 72, 81, and 89), and unknown-risk types (30, 32, 34, 62, 67, 69, 71, 74, 83, 84, 85, 86, 87, 90, and 91) [de Villiers et al., 2004; Munoz et al., 2006; Nobre et al., 2008]. Different prevalence of HPV have been reported in distinct populations and therefore it is important to characterize the most prevalent genotypes in specific population that will improve the clinical approach and also improve the intervention on vaccination programs [Bruni et al., 2010; de Sanjose et al., 2007; de Sanjose et al., 2010].

There are few studies indicating HPV genotypes prevalence between Portuguese populations and therefore we aimed to characterize HPV genotype profile in women from northern region of Portugal and determine the physical status of HPV in cases with HPV-16 infection.

SUBJECTS, MATERIALS, AND METHODS

Study population

This study was performed with exfoliated cervical cells collected from 95 inpatient women with median age of 42±13.1 years old at the Portuguese Institute of Oncology of Porto. All samples were submitted to cytological examination and classified according to the Bethesda classification with the final diagnosis of the women to be confirmed by histological evaluation by experimented pathologists from our institution. Of the 95 patients examined, histological classification revealed 13 normal cervical epithelium (median age 49.0±11.0), 30 cervicitis (median age 44.0±12.8), 24 cervical intraepithelial neoplasia grade 1 (CIN1) (median age 37.5±9.43), 7 cervical intraepithelial neoplasia grade 2 (CIN2) (median age 38.0±9.25), 12 cervical intraepithelial neoplasia grade 3 (CIN3) (median age 45.5±10.8), 4 carcinoma *in situ* (CIS) (median age 48.0±19.5) and 5 invasive cervical carcinomas (ICC) (median age 75.0±20.8). Correlation between histological and cytological exams is described in Table I.

Sample processing

Samples were collected in *ThinPrep*® tubes (Hologic, USA) and stored at room temperature prior processing. A 4ml aliquot was prepared for *digene HC2 High-Risk HPV DNA Test* (QIAGEN, Germany) and 1ml aliquot was used for nucleic acid extraction. Total nucleic acids extraction was performed using *High Pure Viral Nucleic Acid kit* (Roche, Germany), according to manufacturer's instructions, and DNA/RNA quality was assessed by measuring the absorbance at 260/280nm. The presence of amplifiable genomic DNA was also tested with a PCR protocol for amplification of a beta-globin gene with the PCO_3 primer 5' – ACA CAA CTG TGT TCA TAG C – 3' and BGII primer 5' – GTC TCC TTA AAC CTG TCT TG – 3'.

The PCR reaction was performed in a 50µl solution with 1x Taq buffer, 2.0mM $MgCL_2$, 0.2mM DNTP'S, 0.30µM each primer, 1U of Taq DNA Polimerase and 0.2ug of genomic DNA. The amplification conditions were as following: denaturation of DNA template at 95ºC for 3 min, followed by 35 cycles of 94ºC for 1min, 55ºC for 1min, and 72ºC for 1min, and a final extension step at 72ºC for 10min. The amplified fragment of 175 base pairs (bp) was analyzed by electrophoresis in 1.5% (w/v) agarose gels stained with ethidium bromide and visualized under UV light.

HPV characterization

HPV infection was detected by *digene HC2 High-Risk HPV DNA Test* (QIAGEN, Germany) (HC2) at the Virology Service of IPO Porto as part of routine procedures.

HPV DNA was also detected through PCR, using the MY09/11 degenerated primers, which amplify a region of 449–458bp depending on HPV type. These primers amplify a highly conserved region of the HPV L1 gene and are potentially capable of detecting a large number of mucosal HPV types in a single PCR reaction (16).

PCR was performed in a 50µl of reaction mixture with 1x PCR Buffer, 4.0mM MgCl2, 0.2mM DNTP'S, 0.40µM of each primer, 1U of *Taq* DNA polymerase and 0.2ug of DNA. The amplification conditions include an initial denaturation step at 95ºC during 3min, followed by 40 cycles of 94ºC for 45s, 55ºC for 45s, and 72ºC for 1min and a final extension step at 72ºC for 5min. The amplified fragment was analyzed by electrophoresis in 1.5% (w/v) agarose gels stained with ethidium bromide and visualized under UV light.

HPV-positive cases were typed by restriction fragment length polymorphism (RFLP) analysis as described by Nobre et al. 2008 [Nobre et al., 2008]. Each restriction endonuclease reaction was performed in a 20µL final volume reaction, using 5µL of MY09/11 PCR product, 2µL of

10x recommended restriction buffer, and 10units of each restriction endonucleases: PstI (New England BioLabs, R0140S), HaeIII (Fermentas Inc., #ER0151, Canada), DdeI (New England BioLabs, R0175L) and RsaI (New England BioLabs, R0167S). Digestion was performed at 37ºC for 5 hours. The restricted fragments were separated by electrophoresis. The identification of HPV types was executed using the algorithm proposed by Nobre et al, 2008 [Nobre et al., 2008] and HPV genotypes were classified into four groups based on their oncogenic activity: high-risk types (HPV 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59 and 68), probable high-risk types (26, 53, 66, 73, 82), low-risk types (6, 11, 13, 40, 42, 43, 44, 54 and 55, 61, 70, 72, 81 and 89) and types of undetermined-risk (30, 32, 34 and 64, 62, 67, 69, 71, 74, 83, 84, 85, 86, 87, 90, 91, 97, 102, 106) [Munoz et al., 2006; Nobre et al., 2010].

HPV-16 integration

The physical status of HPV16 was analyzed using a multiplex Real-time PCR that allows simultaneous amplification of the E2 and E6 regions [Canadas et al., 2010].

PCR reaction was performed in a 50µl of reaction mixture, including 0.2ug of DNA, 1x Universal Real-Time Master Mix (Applied Biosystems, Foster City, California USA), 30pmol of E2 forward and reverse primers, 6pmol of E2 probe, 3pmol of E6 primers and 1pmol of E6 probe. The amplification conditions include one step at 50ºC for 2min, followed by 45 cycles of 95ºC for 1 min, 55ºC for 1 min and 72ºC for 1min. HPV-16 status classification was based on the principle that, when integration occurs, the E2 gene is partially or totally disrupted while the E6 gene remains intact [Canadas et al., 2010].

Quality Criteria

To ensure the quality of the study we have defined the following quality criteria: 1) inclusion of negative and positive controls in all PCR reactions (as negative controls we have used double distilled water replacing template DNA and as positive control we used a sample from the External Quality Control Panel of the Virology Service for the diagnosis of HPV infection; 2) repetition of 100% of samples for validation of results; and 3) analysis of all results by two independent researchers.

Statistical Analysis

Statistical Analysis was performed using the computer software *IBM® SPSS® (Statistical Package for Social Sciences) Statistics* version 20.0 for Mac. Frequencies among groups were compared, whenever appropriate, by Student's t-test, ANOVA and Chi-square (χ^2) test. Results are considered significantly different when $P<0.05$. The OR (Odds Ratio) and its 95% confidence interval were calculated as a measure of the association between categorical variables.

RESULTS

HPV frequency

Correlation of HC2 results was performed with MY09/11 PCR in 88 samples with adequate quality of DNA (Table II). The presence of inconclusive results was similar in both HC2 and PCR test (n=3 and n=4, respectively). However, we observed 7 positive cases detected by HC2 without amplification in PCR. As expected, the inverse correlation happens in 2 cases of low-risk HPVs. In general, our results suggest that the results obtained by two techniques were consistent (Table II).

Considering results obtained by HC2, HPV infection was detected in 63.7% (58/91) of the cervical specimens (4 cases were considered inconclusive by the test). HPV infection was of 38.5% (5/13) for normal samples, 44.8% (13/29) for cervicitis, 63.6% (14/22) for CIN1, 85.7% (6/7) for CIN2, 100% (12/12) for CIN3, 100% (4/4) for CIS and 100% (4/4) for ICC (Table III).

HPV infection was stratified according to four different age groups (<35, 35–45, 45-55, >55 years old) and results showed that HPV infection was more frequent in women younger than 35 (77.8%) than in women aged 35–45 years old (69%), 45-55 years old (47.8) or older than 50 years old (50%) (Figure 1). Statistical significant differences were found when comparing HPV infection among women younger than 45 and women older than 45 (p=0.021; OR=2.78, 95% CI 1.15-6.73).

Risk-association was estimated by statistical analysis considering that there were no difference between normal and cervicitis cases, which were grouped into a new group (women with no lesion); and CIN1 were categorized as LSIL and CIN2 and CIN3 grouped into HSIL. The analysis revealed a strong association between HPV infection and neoplasia development (OR=5.45; 95%CI 2.09–14.2; P=0.001) when comparing the prevalence of

HPV in all grades of neoplasia (low- and high-grade lesions and carcinoma) versus the reference category (normal and cervicitis). Moreover, statistical significant differences were found when comparing HPV distribution in different individual grades of lesions (Table IV).

All positive cases (n=58) were genotyped in order to characterize the HPV population in our sample (Table III), nevertheless we were not able to genotype a total of 9 cases: 2 normal, 3 cervicitis, 1 CIN1, 1 CIN2 and 2 CIS. Overall, 16 different types were detected: 9 high-risk (HPV16, 18, 31, 33, 39, 52, 56, 58, 59); 4 probable high-risk (HPV53, 66, 82, 83); and 3 low risk (HPV11, 61 and 70). Notably, in the present study, high-risk HPV types were more frequent (85.7%) than probable high-risk HPV types (8.2%) or low-risk (6.1%). The most frequent types of HPV found were HPV16 (single and co-infection, 42.9%) followed by HPV31 (12.2%) and HPV58 (10.2%) (Table V). In our population co-infections were uncommon since we only detected 2 cases with co-infection (HPV16+HPV83 and HPV53+HPV39).

HPV16 integration

We have been able to characterize HPV physical status in 19 out of the 21 positive samples for HPV16. The prevalence of HPV16 integrated forms was of 31.6% (6/19). There were no integrated forms found either in patients with cervicitis and CIN2. The prevalence of HPV-16 integrated forms among CIN1 was of 40.0% (2/5), 33.3% (2/6) in CIN3, 100% (1/1) in CIS and 25.0% (1/4) in ICC (data not shown). Statistical analysis revealed no significant differences in HPV-16 integration distribution among the histological classification (p=0.578) or age groups (p=0.440) (data not shown).

DISCUSSION

HPV infection is one of the most common sexually transmitted infections and a major public health concern [Ho et al., 1998; Moscicki et al., 2001]. Persistent infection with one or more carcinogenic types of HPV may lead to the emergence of intraepithelial lesions, which may progress to high-grade dysplasia or to invasive cervical carcinoma [Woodman et al., 2007].

One of the major problems in HPV characterization studies has been the reliability of cytological classification that commonly introduces biases in the analysis. In our study we have selected 95 patients attended at the Gynecological Service of our institution that have made a confirmatory biopsy after initial cytology. Surprisingly, our results showed that 42.9% of women without lesions (normal histology and cervicitis) of the cervix were positive for HPV infection. This frequency seems to be higher to those found in other studies, which refer that HPV infection among asymptomatic women may vary from 10 to 26% depending on the population[Bruni et al., 2010; de Sanjose et al., 2007; Pista et al., 2011a]. For intraepithelial lesions, including carcinomas, the prevalence of HPV infection was similar to recently described in worldwide studies [Bosch et al., 1993; de Sanjose et al., 2010; Medeiros et al., 2005; Nobre et al., 2010; Smith et al., 2007].

Another common characteristic that is consistently described in literature is the correlation of HPV infection with age distribution, being the younger women most frequently found to be infected. As expected, we observed that HPV prevalence was significantly higher in younger women (p=0.021). This correlation might be explained by exposition to risk factors that are more frequent among young population and also to the development of acquired immunity over time after exposure[Franco and Cuzick, 2008; Silva et al., 2011].

HPV DNA analysis may be performed by several methodologies, and despite no significant differences, in our study HC2 proved to be more sensitive than MY09/11 PCR (data not shown). HC2 is probably the oldest method to detect HPV DNA, approved by Food and Drug

Administration (FDA, USA), and is still considered to be the "gold standard" for HPV DNA detection [Gravitt et al., 2000; Iftner and Villa, 2003].

In order to improve the clinical utility of HPV detection, several methods are being developed to identify the different HPV genotypes. In our study, we have used the genotyping method described by Nobre et al, 2008, which allows a good discrimination of mucosal HPV types, including several HPV types that are underdiagnosed by HC2. Despite the HC2 test has been used to detect 13 high-risk HPV types (HPV 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, and 68) in our study we have found that there might be some cross-reactivity of HC2 HPV DNA probes with others HPV types (HPV 11, 53, 61, 66, 70, 82, and 83) that were detected in this study.

Our study showed that high-risk HPV types are the most frequent, with HPV16 and HPV31 to have higher frequency. Despite HPV18 is the second most common high-risk type associated with cervical cancer, it was not among the most frequents in our population. In fact, HPV16 has been described as the most prevalent type followed by HPV31 and HPV58 in European women, including in Portuguese population [de Sanjose et al., 2007; Medeiros et al., 2005; Nobre et al., 2010; Pista et al., 2011a; Pista et al., 2011b]. Currently underdiagnosed, HPV53 and HPV66 have emerged between the most frequents, and scientific community has been debating is they must be considered probable high-risk or high-risk types [de Sanjose et al., 2007; de Villiers et al., 2004; Medeiros et al., 2005; Nobre et al., 2010; Pista et al., 2011a; Pista et al., 2011b].

Controlling HPV infection is thought to be the key to eradicate cervical cancer. Recent reports demonstrate that through HPV vaccination a promising prevention may be effective toward a reduction of the global burden of cervical cancer in 20 years [Franco and Cuzick, 2008]. An understanding of the epidemiology of HPV genotypes is important to improve screening programs of cervical cancer and to assessment of the impact of HPV16/18 vaccines.

According to several studies, over 70% of all cervical cancers are associated with HPV16 and HPV18 infection and therefore it is expected that the current prophylactic HPV16/18 vaccine will be effective in the prevention of cervical cancer in a significant women [Smith et al., 2007]. However, our data suggests that in our population the existence of other types than HPV16/18 may reduce the impact of HPV vaccination programs in cervical cancer reduction. Thus, it is extremely important to develop epidemiological studies regarding HPV genotype characterization in populations to evaluate the cost-effectiveness of actual HPV vaccines and to predict the impact of new multiple-type vaccines in the near future.

Recent studies have discussed the role of viral integration in cervical cancer development. Viral integration has been described as an indicator of increased risk to malignant progression [Hwang and Shroyer, 2012; Pett and Coleman, 2007; Ramanakumar et al., 2010]. In our study, we have tested for the characterization of HPV16 physical status, the most prevalent genotype. Results revealed that integrated forms are present in different lesions of the cervix whereas women without lesions had only episomal forms. Nevertheless, our study has few cases of HPV16 infection requiring further studies, preferably cohort studies, to follow-up normal and LSIL cases that are infected with HPV in integrated state and evaluate their progression.

In conclusion, although the number of samples was limited and further large-scale epidemiological studies are needed to assess the significance of less common genotypes, this study provides a view on the spectrum of the most common mucosal HPV types in Portuguese women.

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Conflict of interest statement

All authors have contributed to this article and none has any conflict of interest with any competing financial interest for the work described in this paper.

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Table I – Correlation between histological and cytological classifications

		Histological Classification						
		Normal	Cervicitis	CIN1	CIN2	CIN3	CIS	ICC
		n (%)	n (%)	n (%)	n (%)	n (%)	n (%)	n (%)
Cytological Classification	Normal	9 (28.1)	14 (43.8)	8 (25.0)	1 (3.1)	---	---	---
	ASC-US	4 (16.0)	12 (48.0)	5 (20.0)	1 (4.0)	---	---	---
	ASC-H	---	---	2 (100.0)	---	---	---	---
	LSIL	---	4 (22.2)	9 (50.0)	2 (11.1)	3 (16.7)	---	---
	HSIL	---	---	---	3 (23.1)	3 (23.1)	3 (23.1)	1 (7.7)
	CIS/ICC	---	---	---	---	1 (20.0)	1 (20.0)	4 (80.0)
	Total (n=95)	**13 (13.7)**	**30 (31.6)**	**24 (25.3)**	**7 (7.4)**	**12 (12.6)**	**4 (4.2)**	**5 (5.3)**

ASC-US, atypical squamous cells of undetermined significance; ASC-H, atypical squamous cells, cannot exclude high-grade; CIN, cervical intraepithelial neoplasia; CIS, carcinoma *in situ*; ICC, invasive cervical carcinoma; LSIL, low-grade intraepithelial lesions; HSIL, high-grade intraepithelial lesions;

Table II: Correlation between HC2 and PCR results

		PCR		
		Negative	Positive	Inconclusive
		n (%)	n (%)	n (%)
HC2	Negative (n=27)	25 (92.6)	2 (7.4)	----
	Positive (n=57)	7 (12.3)	49 (86.0)	1 (1.8)
	Inconclusive (n=4)	---	2 (50.0)	2 (50.0)
	Total	**32 (36.4)**	**53 (60.2)**	**3(3.4)**

HC2, hybrid capture 2; PCR, polymerase chain reaction

Table III: Distribution of HPV genotypes in study population.

	HPV Positive			
	Total, n (%)	Low-risk, n (%)	High-risk, n (%)	pHigh-risk, n (%)
Normal (n=13)	5 (38.5)	---	2 (66.7)	1 833.3)
Cervicitis (n=29)	13 (44.8)	2 (20.0)	7 (70.0)	1 (10.0)
CIN I (n=22)	14 (63.6)	---	13 (100)	---
CIN II (n=7)	6 (85.7)	1 (20.0)	3 (60.0)	1 (20.0)
CIN III (n=12)	12 (100)	---	11 (91.7)	1 (8.3)
CIS (n=4)	4 (100)	---	2 (100)	---
ICC (n=4)	4 (100)	---	4 (100)	---
Total (n=91)	58 (63.7)	3 (6.1)	42 (85.7)	4 (8.2)

CIN, cervical intraepithelial neoplasia; CIS, carcinoma *in situ*; ICC, invasive cervical carcinoma

Table IV: Prevalence of HPV infection in different lesions.

	HPV Negative	HPV Positive	*P*	OR	95% CI	P^a	OR[a]	95% CI[a]
	n (%)	n (%)						
Normal (n=13)	8 (61.5)	5 (38.5)		1.00	reference		1.00	reference
Cervicitis (n=29)	16 (55.2)	13 (44.8)	0.700	1.30	0.34 – 4.94	0.748	1.25	0.32 – 4.91
Without lesion (n=42)	24 (57.1)	18 (42.9)		1.00	reference		1.00	reference
LSIL (n=22)	8 (36.4)	14 (63.6)	0.114	2.33	0.81 – 6.75	0.455	1.55	0.49 – 4.95
HSIL (n=19)	1 (5.3)	18 (94.7)	<0.001	24.0	2.93 – 196.8	**0.005**	21.3	2.55 – 176.8
CIS/ICC (n=8)	---	8 (100)	0.003	NC	NC	**0.003**	NC	NC
All lesions	9 (18.4)	40 (81.6)	0.001	4.74	1.82 – 12.4	**0.001**	5.45	2.09 – 14.2

LSIL, low-grade intraepithelial lesions; HSIL, high-grade intraepithelial lesions; CIS, carcinoma *in situ*; ICC, invasive cervical carcinoma; a, Adjusted for age; NC, not-computable.

Table V: Distribution of different HPV genotypes

	n (%)
HR HPV (n=40)	
16	20 (40.8)
18	1 (2.0)
31	6 (12.2)
33	2 (4.1)
39	3 (6.1)
52	1 (2.0)
56	1 (2.0)
58	5 (10.2)
59	1 (2.0)
pHR HPV (n=4)	
53	1 (2.0)
66	2 (3.8)
82	1 (2.0)
LR HPV (n=3)	
11	1 (2.0)
61	1 (2.0)
70	1 (2.0)
Co-infection (n=2)	
16 + 83	1 (2.0)
39 + 53	1 (2.0)

HR HPV, High-risk HPV; pHR HPV, Probable High-risk HPV; LR HPV, Low-risk HPV

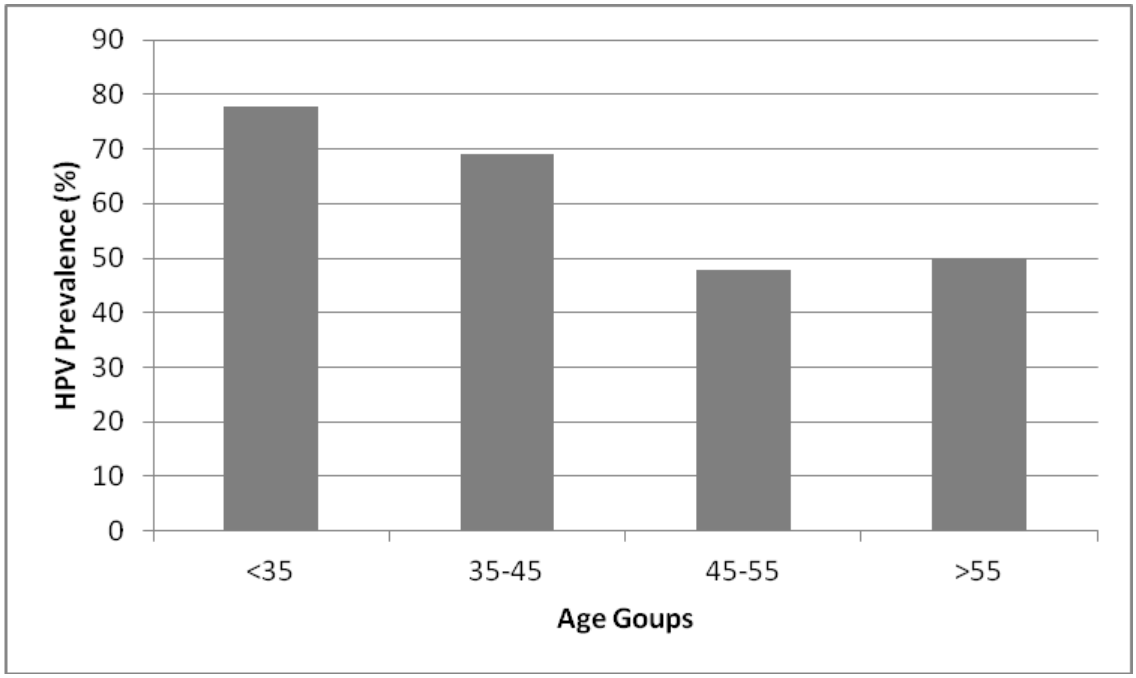

Figure 1: Age-stratified HPV distribution.

CHAPTER IV

miR-34a AND miR-125b EXPRESSION IN HPV INFECTION AND CERVICAL CANCER DEVELOPMENT

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ABSTRACT (202 words)

Background: Clinicians are still demanding for predictive/prognostic biomarkers for HPV infection and cervical lesions progression. Recent studies suggested microRNAs as possible biomarkers of HPV-associated cancers, and therefore we aimed to characterize miR-125b and miR-34a expression in cervical samples.

Methods: miR-125b and miR-34a expression was determined by qRT-PCR methodology in 110 women with different cervical lesions: normal epithelium with (n=20) and without (n=29) HPV infection: LSIL (n=28); HSIL (n=21) and CIS/ICC (n=12).

Results: We observed a two-fold increased miR-125b expression among normal cases with HPV infection ($2^{-\Delta\Delta Ct}$=2.11; p=0.038). Data also showed a trend to down-regulation of miR-25b in LSIL and HSIL cases and a significant decreased expression in women with either CIS or ICC ($2^{-\Delta\Delta Ct}$=0.75; $2^{-\Delta\Delta Ct}$=0.78; $2^{-\Delta\Delta Ct}$=0.33 and $2^{-\Delta\Delta Ct}$=0.23, respectively). miR-34a expression analysis revealed an increased expression among women with normal cervix and HPV infection ($2^{-\Delta\Delta Ct}$=1.69; p=0.049) but no significant change was observed for LSIL, HSIL or CIS/ICC.

Conclusion: This is the first study to characterize the expression of miR-125b and miR34a in cervical samples. Results showed that while miR-34a expression remains constant, miR-125b expression is significantly changed in the different cervical lesions and its levels should be further investigated as possible predictive/prognostic biomarkers using a non-invasive approach.

Key-words:

Cervical Cancer, Biomarkers, miR-125b, miR34a

INTRODUCTION

Cervical cancer is the third most common cancer among women with approximately 530 000 new cancer cases and 275 100 deaths each year (Jemal *et al*, 2011; Pinheiro *et al*, 2003). Persistent infection by human papillomavirus (HPV) has been considered the etiological cause of squamous intraepithelial lesions of the cervix that may develop into high-grade dysplasia or to invasive carcinoma. High-risk HPVs (HR-HPV) are recognized a necessary but non-sufficient condition for the development of cervical cancer (zur Hausen, 1991; zur Hausen, 2009a; Zur Hausen, 2009b; Zur Hausen & Rosl, 1994). The majority of HPV infections are asymptomatic and are efficiently controlled by the host immune system, therefore, the outcome of HPV infection is variable (Woodman *et al*, 2007). Nowadays, screening programs of cervical cancer/lesions are based in cytology exam (Pap smear test) and HPV DNA detection. However, clinicians still demand for the identification of useful predictive/prognostic biomarkers for HPV infection and progression of lesions.

Several studies have been performed to identify potential biomarkers for HPV infection outcome considering distinct events of HPV-related carcinogenesis and cellular modifications caused by HPV infection (Hwang & Shroyer, 2012). Recently, microRNAs (miRNAs), non-coding RNAs with proximally 18–25 nucleotides in length, have been proposed as optimal markers for cancer approach. miRNAs are responsible for modulate gene expression by binding to complementary segments present in the UTR of the mRNAs of protein coding genes leading to the suppression of translation and/or degradation of messenger RNA (mRNA) degradation (Melo & Esteller, 2011). miRNAs are thought to potentially target up to one-third of human coding genes managing cellular activity, including proliferation, differentiation and apoptosis (Malumbres, 2012; Melo & Esteller, 2011). These molecules have been described as key regulators including cancer (Kong *et al*, 2012). In fact, several studies have been addressing the impact of miRNAs in tumor

development either can act as oncogenes or tumor suppressor genes depending on neoplasia and number of miRNAs involved in human cancer has been growing (Malumbres, 2012).

Recent studies have suggested microRNAs as possible markers for the occurrence and development of the HPV-associated cancers, including cervical cancer (Pereira *et al*, 2010). Moreover, studies have been described several interactions between microRNAS and HPV oncoproteins. Specific microRNAs have been located in cancer-related genomic regions, which include fragile sites at or near HPV integration sites. Differences in tumor-specific microRNA signatures might be an important tool to distinguish different lesions of the cervix (He *et al*, 2007a; He *et al*, 2007b; Martinez *et al*, 2008).

The aim of this study was to characterize the expression of miR-34a and miR-125b in different cervical lesions and cancer, including normal cervical epithelium with or without HPV infection and to evaluate its impact as predictive/prognostic biomarkers of cervical cancer and HPV infection.

SUBJECTS, MATERIALS, AND METHODS

Study population

The study was performed in exfoliated cervical cells collected from 110 inpatient women with median age of 39±12.2 years old at the Portuguese Institute of Oncology of Porto. All samples were submitted to cytological examination classified according to the Bethesda classification, and confirmed by histological evaluation by experimented pathologists from our institution. The population of the study is constituted by 49 women with normal cytologies, 26 with low-grade intraepithelial squamous lesions (LSIL), 21 with high-grade intraepithelial squamous lesions (HSIL), 7 with carcinomas *in situ* (CIS) and 4 with invasive cervical carcinomas (ICC).

Sample processing

Samples were collected in *ThinPrep*® tubes (Hologic, USA) and stored at room temperature prior processing: a 4mL aliquot was used for *digene HC2 High-Risk HPV DNA Test* (QIAGEN, Germany); and 1 mL was used for total nucleic acids extraction using *High Pure Viral Nucleic Acid kit* (Roche, Germany).

HPV infection

HPV infection was detected by *digene HC2 High-Risk HPV DNA Test* (QIAGEN, Germany) (HC2) at the Virology Service of IPO Porto as part of routine procedures. HC2 test detect 13 high-risk HPV (HR-HPV) types: 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, and 68. HPV infection was detected in 65.4% of all cervical specimens, with a prevalence of 40.8% (20/49) for normal cytology, 73.1% (19/26) for LSIL, 95.2% (20/21) for HSIL, 100% (7/7) for CIS and 100% (4/4) for ICC.

miRNA analysis

miR-125b, miR-34a and miR-23a were analysed using two-step real-time PCR protocols using TaqMan MicroRNA Assays: has-miR-125b-5p_000449; has-miR-34a-5p_000426; has-miR-23a-3p_000399, respectively (Applied Biosystems, Foster CA, USA).

Reverse transcriptase reactions were performed using TaqMan MicroRNA Reverse Transcription Kit (PN 4366596; Applied Biosystems, Foster CA, USA) in a 15uL of total volume reaction mix with: 7uL of master mix containing 1x RT buffer, 1.0mM of total dNTPs, 50U MultiScribe Reverse Transcriptase Enzyme and 0.25U of RNase inhibitor; 3uL of RT primers (Applied Biosystems, Foster CA, USA); and 5uL of RNA sample. The amplification conditions were as following: annealing at 16ºC for 30 min, extension at 42 ºC for 52 min and RT inactivation at 85ºC for 10 min. All reverse transcriptase reactions included two no-template controls (as negative controls we used double distilled water replacing template RNA).

qPCRs were performed in independent reactions on an Applied Biosystems 7300 Real Time PCR System (Applied Biosystems, Foster CA, USA) with a 20µl final volume mixture containing 1× TaqMan® Universal PCR Master Mix (Applied Biosystems, Foster City, California USA), 1x MicroRNA Assay (Applied Biosystems, Foster City, California USA) and 2 uL cDNA (RT product). Thermal cycling conditions were: 10 min. at 95°C followed by 45 cycles of 15 sec. at 95°C and 1 min. at 60°C. All reactions were run in duplicate.

Data analysis

The endpoint of qPCR data is the threshold cycle (*C*t). The Ct is defined as the fractional cycle number at which the fluorescence passes the fixed threshold.

The relative quantification of miRNA expression was analyzed using Livak method (also known as $2^{-\Delta\Delta Ct}$ method). In this method, Cts from the target miRNAs (miR-125b and miR-

34a) in both test and reference cases were adjusted in relation to the Ct of a normalizer miRNA (miR-23a) resulting in ΔC_t. The reference group in this study was women with normal cytology and without HPV infection. The difference between the ΔCt of cases and ΔCt reference gives the ΔΔCt value, which is incorporated to determine the fold difference in expression.

Statistical Analysis

Statistical Analysis was performed using the computer software *IBM® SPSS® (Statistical Package for Social Sciences) Statistics* version 20.0 for Mac. All cytologyc groups were compared, whenever appropriate, by Student's t-test, ANOVA and Chi-square (χ^2) test. Results are considered significantly different when $P<0.05$. Receiver operating characteristic (ROC) curves were used to assess the predictability of cervical disease progression.

RESULTS

qPCR analysis

qPCR results are shown in Table I where we describe, for all groups, the mean Ct, standard deviation, Ct range and the Variation Coefficient (VC). Firstly, we observed that qPCR was able to detect both low and high quantities of the miRNAs (overall ranging from 24.53 to 41.35). Additionally, the VC for all miRNAs was bellow 10%, which reveals that the variation of Ct within samples was very low. These data also show that the *High Pure Viral Nucleic Acid kit* (Roche, Germany) was able to extract efficiently miRNA from this type of samples and that it could be used efficiently for future analysis.

miRNAs expression

Expression of selected miRNAs was determined for all samples, grouped according to cytological result, and normalized with a control group of women with normal cytology and without HPV infection (Table II).

Considering the results of miR-125b expression (Table II and Fig1) there are interesting data: firstly, we observed a two-fold (2.11 relative expression) increase among normal cases with HPV infection (p=0.038); then, and despite there is no statistical significance, we also found a decrease of miR-125b expression for LSIL or HSIL (0.75 and 0.78 relative expression, respectively); finally, the analysis revealed that miR-125b expression was significantly decreased in women with either CIS or ICC (0.33 and 0.23 relative expression, respectively).

In contrast to miR-125b, no differences in miR-34a expression were observed for the majority of study groups (Table II and Fig2). Despite the increase of miR-34a expression (1.69 relative expression) among women with normal cervix and HPV-infected (p=0.049), miR-34a is almost stably expressed in LSIL, HSIL or CIS/ICC (1.01, 1.32 and 1.06 relative expression, respectively). Additionally, we observed that when considering the CIS and ICC isolated we

observed that it seemed to be significant differences in relative expression (0.75 and 0.78 relative expression, respectively), although these were not statistically significant.

DISCUSSION

In the late decade miRNAs have been described as possible biomarkers of cancer development by regulating cellular behavior. Actually, miRNAs expression and biological functions are highly influenced by cellular context, probably due to the differential expression of their target mRNAs (Malumbres, 2012). In fact, the miRNAs expression varies from tissue to tissue and several studies attested that they are differentially expressed in abnormal tissue when compared with normal adjacent tissues. Moreover, miRNAs are thought to be highly stable when compared with mRNA and therefore their detection is relatively easy and reproducible, therefore suggesting that miRNAs might be good candidates for biomarkers and diagnostic tools in oncology.

Recent studies have been developed to identify proof-valuable biomarkers for HPV infection outcome considering the cellular modifications caused by HPV infection and the cascade of distinct events of HPV carcinogenesis (Hwang & Shroyer, 2012). Literature have shown differential miRNA expression in cervical lesions and cancer tissues and also revealed potential interactions of miRNAs in HPV-associated carcinogenesis (Pereira *et al*, 2010). In our study, we investigated the expression of two miRNAs (miR-125b and miR-34a) that have been described as interacting in crucial steps of cervical cancer development.

Our study is the first using qRT-PCR methodology to evaluate the expression of miRNAs in exfoliated cervical cells with high sensitivity and specificity. Nevertheless, we are aware that our study has limited number of samples mainly in CIS/ICC group and therefore it would be useful to develop further studies with increased number of samples. Moreover, the results could be significantly confirmed if we could access the biopsy sample of each patient and additionally improve the study with p53 expression either in biopsy or cytological samples.

miR-125b has been described as involved in different cellular processes such as inflammation, cell proliferation and cell cycle regulation and therefore its action can be either as oncogene or

tumor suppressor depending on cell type (Jia *et al*, 2012; Le *et al*, 2009; Tili *et al*, 2008; Zhang *et al*, 2011). Our results start by demonstrating that miR-125b expression is increased in normal cervix infected by HPV, while the relative expression seems to decrease as lesions progress (Table II). According to literature, an increased expression of miR-125b shortly after HPV infection can be explained by interaction with viral proteins (Nicolete *et al*, 2012; Witwer *et al*, 2012). In fact, a recent study reveals that there is a strong homology between miR-125b and the HPV-L2 and therefore it is expected an increased expression of miR-125b shortly after viral infection as response to HPV proliferative with expression of HPV-L2 (Nuovo *et al*, 2010). Additionally, Nuovo and colleagues showed that after persistent infection, HPV-L2 can induce inactivation of mir-125b and this event seems to be associated with cytological changes of the koilocytes (Nuovo *et al*, 2010). Literature also refers that since miR-125b is a negative regulator of p53 (Le *et al*, 2009; Zhou *et al*, 2010), its expression would allow a more effective suppression of p53-pathways and therefore contribute to abnormal transformation of infected cells (Sousa *et al*, 2007; Woodman *et al*, 2007).

miR-34a activity is considered as tumor suppressor and has been reported as down-regulated in several cancers (Hermeking, 2012). This miRNA is implicated in the p53-network and has been described as direct target of p53: when p53 is activated it induces the transcription of miR-34a which is able to target several molecules involved in cellular transformation and carcinogenesis (Bommer *et al*, 2007; Chang *et al*, 2007; Corney *et al*, 2007; He *et al*, 2007a; He *et al*, 2007b; Hermeking, 2012). Theoretically, in HPV-associated carcinogenesis, the expression of HPV-E6 will down-regulate the expression of miR-34a by targeting p53 to degradation thought the ubiquitin-proteasome system (Hermeking, 2012; Li *et al*, 2010; Sousa *et al*, 2007; Sousa *et al*, 2011). Therefore, it is expected that miR-34a deregulation occur after HPV infection, probably after viral genome integration into host's genome. Li and colleagues

reported that inhibition of miR-34a expression is observed in precancerous lesions and probably represents an early-onset event in cervical cancer development (Hermeking, 2012; Li *et al*, 2010). In fact, several studies have been showed that levels of expression of miR-34a are significantly reduced in both cancer tissues and cervical cell lines, compared with normal tissues (Li *et al*, 2010; Wang *et al*, 2009). Although we have found an initial increase of miR-34a expression in normal cells infected by HPV, we did not observe significant differences in relative expression when considering LSIL, HSIL or CIS/ICC. The increase of miR-34a expression in HPV infected cells is probably explained by the activation of cellular repair mechanisms after viral infection that would activate p53-pathways and therefore induce miR-34a expression. Nevertheless, results do not corroborate prior reports on miR-34a and cervical cancer and therefore we conclude that miR-34a expression profile in HPV infection and cervical lesions/cancer remains unclear.

In conclusion, our study revealed that miR-125b expression is up-regulated in normal tissues after HPV infection, and decreases significantly as cervical lesions progress to invasive carcinoma. In fact ROC revealed significant sensitivity and specificity for CIS/ICC diagnosis (data not shown). These results suggest that miR-125b could probably be used as useful tool for cervical lesions diagnosis/prognosis using a non-invasive approach, though this observation needs to be complemented with further studies.

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Conflict of interest statement

All authors have contributed to this article and none has any conflict of interest with any competing financial interest for the work described in this paper.

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Table I: qPCR data analysis

	miR125b		miR34a		miR23a	
	Mean Ct ± sd (range)	VC	Mean Ct ± sd (range)	VC	Mean Ct ± sd (range)	VC
No Lesion (no HPV) (n=29)	28.43 ± 2.48 (24.60 – 36.51)	0.09	34.14 ± 1.82 (30.07 – 37.95)	0.05	34.94 ± 1.72 (32.53 – 39.49)	0.05
No Lesion (HPV+) (n=20)	28.06 ± 2.20 (24.53 – 32.07)	0.08	34.09 ± 1.58 (31.18 – 37.10)	0.04	35.65 ± 2.58 (32.30 – 41.35)	0.07
LSIL (n=28)	29.34 ± 2.12 (25.55 – 34.34)	0.07	34.60 ± 1.71 (31.49 – 38.15)	0.05	35.42 ± 2.04 (31.85 – 40.97)	0.06
HSIL (n=21)	29.06 ± 1.81 (26.48 – 32.71)	0.06	34.01 ± 1.22 (32.29 – 36.89)	0.04	35.21 ± 1.73 (32.32 – 39.13)	0.05
CIS/ICC (n=12)	29.39 ± 2.67 (26.08 – 33.85)	0.09	33.22 ± 1.89 (28.67 – 35.86)	0.06	34.10 ± 1.29 (31.79 – 36.92)	0.04
CIS (n=7)	29.48 ± 2.66 (26.20 – 33.85)	0.09	34.28 ± 1.03 (32.54 – 35.86)	0.03	34.40 ± 1.65 (31.79 – 36.92)	0.05
ICC (n=5)	29.27 ± 2.99 (26.08 – 33.11)	0.10	31.73 ± 1.87 (28.67 – 33.34)	0.06	33.68 ± 0.35 (33.33 – 34.33)	0.01

Ct, cycle threshold; Sd, Standard Deviation; VC, variation coefficient; LSIL, Low-grade intraepithelial lesions; HSIL, High-grade intraepithelial lesions; CIS, carcinoma *in situ*, ICC, Invasive cervical carcinoma.

Table II: Expression profile data for miR-125b and miR-34a in cervical lesions

	miR125b			miR34a		
	ΔCt ± sd (range)	$2^{-\Delta\Delta Ct}$	t-test	ΔCt ± sd (range)	$2^{-\Delta\Delta Ct}$	t-test
No Lesion (no HPV) (n=29)	-6.51 ± 1.73 (-10.59 – -0.77)	Reference		-0.80 ± 1.16 (-2.82 – 1.67)	Reference	
No Lesion (HPV+) (n=20)	-7.59 ± 1.77 (-10.79 – -4.28)	2.11	**0.038**	-1.56 ± 1.47 (-4.30 – 0.54)	1.69	**0.049**
LSIL (n=28)	-6.09 ± 1.44 (-9.86 – -3.94)	0.75	0.320	-0.82 ± 1.81 (-4.26 – 2.45)	1.01	0.955
HSIL (n=21)	-6.15 ± 1.83 (-9.10 – -2.30)	0.78	0.481	-1.20 ± 1.74 (-5.32 – 1.65)	1.32	0.335
CIS/ICC (n=12)	-4.71 ± 2.22 (-8.24 – -1.13)	0.29	**0.008**	-0.88 ± 1.62 (-4.87 – 0.75)	1.06	0.156
CIS (n=7)	-4.92 ± 2.00 (-8.24 – -3.07)	0.33	**0.042**	-0.12 ± 0.84 (-1.47 – 0.75)	0.62	0.074
ICC (n=5)	-4.41 ± 2.70 (-7.46 – -1.13)	0.23	**0.028**	-1.94 ± 1.92 (-4.87 – -0.26)	2.20	0.857

Ct, cycle threshold; Sd, Standard Deviation; LSIL, Low-grade intraepithelial lesions; HSIL, High-grade intraepithelial lesions; CIS, carcinoma *in situ*, ICC, Invasive cervical carcinoma.

Figure 1: Expression profile of miR-34a in different cervical lesions

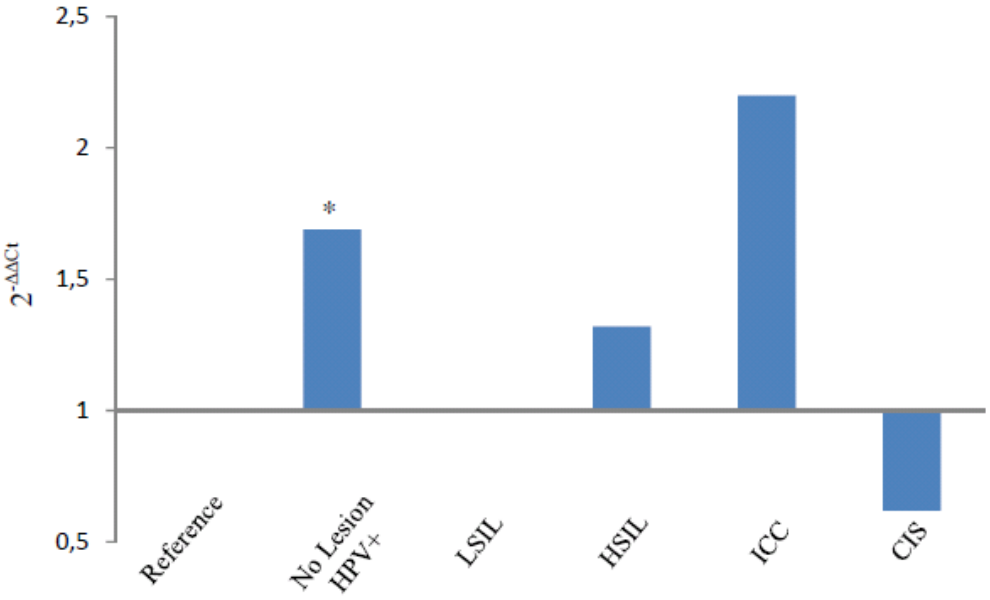
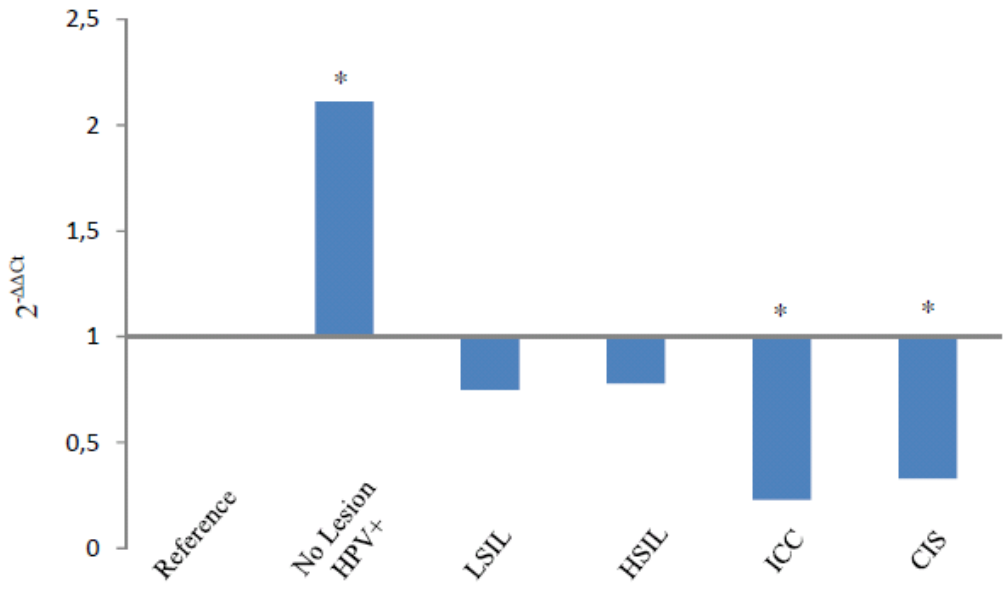


Figure 2: Expression profile of miR-125b in different Cervical lesions



GENERAL DISCUSSION AND FUTURE STUDIES

GENERAL DISCUSSION AND FUTURE STUDIES

Clinical and subclinical HPV infections are the most common sexually transmitted infections in the world and this infection has been established as the cause of genital warts, squamous intraepithelial lesions of the cervix and other types of anogenital lesions [zur Hausen, 1991; zur Hausen, 2009a; Zur Hausen, 2009b; Zur Hausen and Rosl, 1994]. However, only certain types of HPVs are able to induce cervical cancer development In fact, HPV can be classified into high-risk types, which are frequently associated with the development of epithelial lesions of the cervix, and low-risk types, which are found mainly in genital warts and benign lesion [de Villiers et al., 2004; Munoz et al., 2006; Nobre et al., 2008].

Portugal has a relatively high incidence rate for cervical cancer and one of the highest mortality rates in the developed countries, mainly due to a limited screening program for cervical cancer [Jemal et al, 2011]. Recently, a vaccination program against HPV infection was included in the National Program of Vaccination and therefore it is expected a decrease in the prevalence of HPV infection among young.

Distribution of HPV genotypes has changed in population in worldwide, hence, and epidemiological studies regarding prevalence of HPV types should to be performed to characterize their frequency. Our results showed that HPV16 is the most frequent types among Portuguese women followed by HPV58, 39, 53 and 66. Surprising, HPV18 proved to be a rare genotype in our population. Although the distribution of HPV genotypes have been evaluated in a small number of women, our data should be included in future approaches, mainly in the decision of future vaccination programs in Portugal.

Integration of viral genome into host´s genome has been considered a crucial event in HPV-associated carcinogenesis and some authors have been proposed that physical status of HPV genome should be a considered as prognostic biomarker of cervical lesions [Hwang et al, 2012]. Different methodologies have been described to determine physical status of HPV genotypes and therefore the variability could be considered a potential disadvantage. In our study we determined the viral physical status only in HPV16 positive cases (the most prevalent HPV type) and the most significant data was that it was possible to detect integrated forms in early lesions of the cervix. Hence, further studies are required, preferably, cohort studies, to follow-up normal and LSIL cases that are infected with HPV in integrated state and evaluate their progression.

MicroRNAs play a fundamental role in human biology and recent studies have suggested these molecules as possible markers for the occurrence and development of the HPV-associated cancers [Melo and Esteller, 2011; Martinez et al, 2008]. Differences in tumor-specific microRNA signatures have been proposed to distinguish different lesions, although conclusions are still unclear. Several studies have been performed determining the expression of different miRNAs in cervical tissues and evaluating their value as biomarkers. We focused this study in two miRNAs (miR-125b and miR-34a) that play a key role in distinct cellular processes. Scientific literature reveals that HPV proteins seem to interact with both miR-34a and miR-125b changing its expression and promoting viral infection and cervical cancer development [Hermeking, 2012; Li et al., 2010; Tili et al., 2008; Le et al, 2009 Zhang et al., 2011].

miR-34a has been included as active member of the p53-network, mainly because it was described as direct target of p53. The activation of p53 leads to miR-34a transcription and consequently to the suppression several molecules involved in cellular transformation and carcinogenesis [Bommer et al., 2007; Chang et al., 2007; Corney *et al.*, 2007; He *et al.*, 2007a; He et al., 2007b].

Our results regarding miR-34a expression in cervical cells revealed an increase expression in normal cervix when infected by HPV; however, no significant difference was observed for LSIL, HSIL or CIS/ICC. This is the first study to indicate that levels of miR-34a remain constant during cervical carcinogenesis. According to the literature, it was expected that after HPV infection the expression of HPV-E6 would target p53 to degradation and therefore miR-34a levels should be decreased. Thus, we think that expression of p53 should be evaluated to correlate with miR-34a levels and understand if there are other mechanism that controls miR-34a expression.

Considering miR-125b, our study revealed that its expression is up-regulated in normal tissues after HPV infection, and decreases significantly as cervical lesions progress to invasive carcinoma. These results are in agreement with a previous *in vitro* study that described an interaction between miR-125b and HPV-L2 a possible role on the initial steps of cervical carcinogenesis, including inflammation and immune response against HPV infection. Our results suggest that miR-125b could probably be used as useful tool for cervical lesions diagnosis/prognosis using a non-invasive approach, though this observation needs to be complemented with further studies.

The role of microRNAs in cervical cancer development is still unclear and studies reporting expression of microRNAs in cervical tissues are needed to clarify this mechanism. A better understanding of the microRNAs in cervical cancer may not only reveal novel pathogenic mechanism of HPV but also identity potential targets for cervical cancer therapy. Additionally, specific miRNA expression profile would be useful as a biomarker for cancer and viral infection.

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