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Ana Sofia Leal de Vilhena Portela de Carvalho

Clinicopathologic significance of ERCC1 expression in breast cancer

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Clinicopathologic significance of ERCC1 expression in breast cancer

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Área do Projeto: Anatomia Patológica

Título do Projeto: Clinicopathologic significance of ERCC1 expression in breast cancer

Resumo: The excision repair cross-complementation 1 (ERCC1) enzyme plays an essential role in the nucleotide excision repair pathway and is associated with resistance to platinum-based chemotherapy in different types of cancer. The aim of the present study was to evaluate the clinicopathologic significance of ERCC1 expression in breast cancer patients. We used immunohistochemical analysis to assess ERCC1 expression in a tissue microarray from 135 breast carcinomas. This was correlated with clinicopathologic factors and outcome data. The clinicopathologic features and immunohistochemical markers of the tumors were compared using the chi-square and the Fisher's exact test. The Kaplan-Meier method was used to analyze overall and disease-free survival. ERCC1 expression analysis was available for 109 cases. In this group, 58 (53.2%) were positive for ERCC1. ERCC1-positive expression was correlated with smaller tumor size (P = 0.007) and with positivity for ER (estrogen receptor) (P = 0.040), but no correlation was found with other clinicopathological features or biomarkers studied. ERCC1 did not correlate with the overall and disease-free survival rates. Although not statistically significant, the majority (72.7%) of special histological types of invasive breast carcinomas was positive for ERCC1 compared to invasive ductal carcinomas, which were ERCC1-positive in 51.1% of the cases. Similarly, triple negative breast cancers (TNBC) were more frequently negative for ERCC1 (61.5% of the cases) compared to the non-TNBC (41.5%). In conclusion, ERCC1 expression correlated significantly with favorable prognostic factors, such as smaller tumor size and ER-positivity, suggesting a possible role for ERCC1 as a predictive and/or prognostic marker in breast cancer.

Palavras-chave: excision repair cross-complementation 1, breast cancer, breast cancer molecular subtypes, immunohistochemistry.

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Resumo: A enzima "excision repair cross-complementation 1" (ERCC1) desempenha um papel essencial na via de reparação do DNA por excisão de nucleotídeos e está associada a resistência à quimioterapia com compostos derivados da platina em diferentes tipos de cancro. O presente estudo pretendia avaliar o significado clinicopatológico da expressão de ERCC1 em doentes com cancro da mama. Foi utilizada análise imunohistoquímica para avaliar a expressão de ERCC1 num "tissue microarray" de 135 carcinomas da mama. Estes dados foram correlacionados com fatores clinicopatológicos e com o prognóstico dos doentes. As características clinicopatológicas e os marcadores imunohistoquímicos dos tumores foram comparados com o teste do qui-quadrado e o teste exato de Fisher. O método de Kaplan-Meier foi utilizado para avaliar a sobrevida global e a sobrevida livre de doença. A análise da expressão de ERCC1 estava disponível para 109 casos. Neste grupo, 58 (53.2%) foram positivos para ERCC1. A expressão positiva de ERCC1 foi correlacionada com tumores com dimensões inferiores a 2.0 cm (P = 0.007) e com positividade para os recetores de estrogénios (RE) (P = 0.040), mas não houve correlação com as restantes características clinicopatológicas ou biomarcadores estudados. A expressão de ERCC1 não teve correlação com as taxas de sobrevida global e livre de doença aos 5 anos. Embora não sendo estatisticamente significativo, a maioria (72.7%) dos tipos histológicos especiais de carcinomas da mama foi positiva para ERCC1 quando comparada com carcinomas ductais, os quais foram positivos para ERCC1 em 51.1% dos casos. Da mesma forma, os cancros da mama triplo-negativos foram mais frequentemente negativos para ERCC1 (61.5% dos casos) comparados com os não-triplo-negativos (41.5%). Em conclusão, a expressão de ERCC1 correlacionou-se de forma significativa com fatores de prognóstico favoráveis, tais como tumores de pequenas dimensões e com positividade para os RE, sugerindo, assim, um possível papel da enzima ERCC1 como marcador preditivo e/ou de prognóstico no cancro da mama.

Palavras-chave: excision repair cross-complementation 1, cancro da mama, subtipos moleculares de cancro da mama, imunohistoquímica

Clinicopathologic significance of ERCC1 expression in breast cancer

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Abstract

The excision repair cross-complementation 1 (ERCC1) enzyme plays an essential role in the nucleotide excision repair pathway and is associated with resistance to platinum-based chemotherapy in different types of cancer. The aim of the present study was to evaluate the clinicopathologic significance of ERCC1 expression in breast cancer patients. We used immunohistochemical analysis to assess ERCC1 expression in a tissue microarray from 135 breast carcinomas. This was correlated with clinicopathologic factors and outcome data. The clinicopathologic features and immunohistochemical markers of the tumors were compared using the chi-square and the Fisher's exact test. The Kaplan-Meier method was used to analyze overall and disease-free survival. ERCC1 expression analysis was available for 109 cases. In this group, 58 (53.2%) were positive for ERCC1. ERCC1-positive expression was correlated with smaller tumor size (P = 0.007) and with positivity for ER (estrogen receptor) (P = 0.040), but no correlation was found with other clinicopathological features or biomarkers studied. ERCC1 did not correlate with the overall and disease-free survival rates. Although not statistically significant, the majority (72.7%) of special histological types of invasive breast carcinomas was positive for ERCC1 compared to invasive ductal carcinomas, which were ERCC1-positive in 51.1% of the cases. Similarly, triple negative breast cancers (TNBC) were more frequently negative for ERCC1 (61.5% of the cases) compared to the non-TNBC (41.5%). In conclusion, ERCC1 expression correlated significantly with favorable prognostic factors, such as smaller tumor size and ER-positivity, suggesting a possible role for ERCC1 as a predictive and/or prognostic marker in breast cancer.

Keywords excision repair cross-complementation 1, breast cancer, breast cancer molecular subtypes, immunohistochemistry

Introduction

Breast cancer is a heterogeneous disease with various histological types of tumors and different clinical behavior. The molecular classification of breast cancer differentiates, at least, three subgroups of tumors: the luminal subtype with cells expressing estrogen receptors (ER) and ER-related genes; the human epidermal growth factor receptor 2 (HER2)-overexpressing subtype; and the basal-like subtype associated with the expression of basal cell markers [1-3]. For the clinical management of breast cancer, a useful manner of defining the molecular subtypes is the classification of tumors using immunohistochemistry or *in situ* hybridization techniques. Immunohistochemical expression of ER and/or progesterone receptors (PR) characterizes luminal tumors, and HER2-overexpressing subtype is defined by overexpression and/or amplification of HER2. Breast tumors that do not express hormone receptors (ER, PR) nor HER2 overexpression and/or amplification are classified as triple negative breast cancers (TNBC) [4]. The vast majority of TNBC are of basal-like phenotype, but this group of cancers also encompasses tumors without the expression of basal markers, including the molecular apocrine and claudin-low tumors [5].

Well-established targeted therapies are available for breast cancers positive for hormone receptors or with a HER2-positive status. Endocrine therapy with tamoxifen, an ER modulator, or with aromatase inhibitors is advocated for breast cancers that express ER, and anti-HER2 therapy using trastuzumab, a monoclonal antibody against HER2, or lapatinip, an inhibitor of the tyrosine kinase activity of HER2, for those tumors with overexpression and/or amplification of HER2 [6]. In contrast, targeted therapies for TNBC are not completely validated and the main treatment for this group of tumors is the use of chemotherapy, including platinum salts, isolated or in combination with other chemotherapy agents [7, 8].

Platinum-based chemotherapy is used in a variety of malignant diseases, including tumors from ovary, testes, lung, cervix, colon, and bladder. Currently, there are three platinum compounds more commonly used, namely cisplatin, carboplatin, and oxaliplatin. Platinum-based drugs

provoke the formation of platinum-DNA adducts leading to changes in the helical structure of the DNA molecule [9, 10]. The distortion of the DNA molecule results in the inhibition of transcription and replication, leading to cell death. DNA adducts are recognized and repaired by the nucleotide excision repair (NER) pathway, including those caused by platinum compounds [9-11]. The excision repair cross-complementation group 1 (ERCC1) is a nuclease that plays an essential role in the NER pathway: ERCC1 forms a heterodimer with xeroderma pigmentosum complementation group F (XPF) protein, and the complex ERCC1-XPF executes the excision of the damaged DNA [9-12]. Therefore, the integrity of the NER pathway is an important predictor of platinum-based chemotherapy resistance [9, 12].

Low levels of ERCC1 are correlated with *in vitro* sensitivity to cisplatin in malignant cell lines from cervical cancer [13], testicular cancer [14], and malignant effusions collected from patients with gastric, gynecological and non-small cell lung cancer (NSCLC) [15]. Retrospective clinical studies have shown an association between high levels of ERCC1 mRNA or protein expression and resistance to platinum-based chemotherapy in different types of advanced cancer, including gastric [16] and colorectal cancer [17, 18], NSCLC [19, 20], urinary tract cancer [21, 22], and head and neck squamous cell carcinoma [23].

There are few studies regarding the expression of ERCC1 in breast cancer. Some studies showed that the expression of ERCC1 is particularly lower in TNBC [24, 25]. On the other hand, increased expression of ERCC1 has been positively correlated with features related to a better prognosis in breast cancer such as patient age > 50 years old, lower T stage, nodal negativity, and ER positivity [26]. There are also few studies regarding patients with breast cancer that were treated with platinum-based regimens. Shao et al. (2010) [27] observed an association between higher ERCC1 expression and shorter progression-free survival in patients treated with paclitaxel plus cisplatin. Another study showed that ERCC1 negative breast tumors were associated with a higher pathological complete remission rate in patients treated with paclitaxel plus carboplatin in neoadjuvant chemotherapy [28].

The aim of this study was to analyze the association between clinicopathologic features and the immunohistochemical expression of ERCC1 in a series of patients with breast cancer. We also analyzed the prognostic significance of ERCC1 in this group of patients.

Materials and Methods

Patients' characteristics

All 135 enrolled patients were cases of primary operable invasive breast cancer. Patients' clinical history data was acquired from the files of the Department of Pathology, Hospital do Divino Espírito Santo, Azores, Portugal. The patients' age ranged from 24 to 60 years. The histological diagnosis of the formalin-fixed paraffin-embedded sections was confirmed by three pathologists as follows: 117 invasive ductal carcinomas (86.7%), 4 invasive lobular carcinomas (3.0%), 1 invasive mixed (mucinous and ductal) breast carcinoma (0.7%) and 13 invasive breast carcinomas of special histological types (9.6%). The latter group included 4 invasive mucinous carcinomas (3.0%), 4 invasive cribriform carcinomas (3.0%), 2 invasive papillary carcinomas (1.5%), 1 invasive tubular carcinoma (0.7%), 1 invasive medullary carcinoma (0.7%), and 1 micropapillary carcinoma (0.7%).

Multiple clinicopathologic and molecular characteristics were obtained, including age, tumor size, histological type, histological grade, lymph node status, TNM stage, the Nottingham Prognostic Index (NPI), tumor molecular subtype (as defined by the immunohistochemical expression of ER, PR, and HER2), and the immunohistochemical expression of Ki-67 index, epidermal growth factor receptor (EGFR), cytokeratin 5 (CK5), P-cadherin, and vimentin (Table 1).

Follow-up ranged from a minimum of 5 months to a maximum of 117 months (median 77.5 months). Disease-free survival (DFS) time was calculated as the duration from the date of surgery to the date of documented disease progression (breast-cancer-derived relapse/metastasis) or the date of the last follow-up. Overall survival (OS) time was calculated as the duration from the date of diagnosis to the date of death or last contact.

This study was conducted according to the Portuguese regulative law for the usage of biological specimens from tumor banks. In consequence, the samples can exclusively be used for research purposes in the context of retrospective studies.

Tissue microarray

A tissue microarray composed of duplicate cores of representative areas of the tumors (2 mm in diameter) deposited in a paraffin block was developed in accordance with previous work (tissue microarray builder ab1802; Abcam, Cambridge, UK) [29, 30]. Normal breast tissue cores were used as controls and included in the paraffin block.

Immunohistochemical study

Immunohistochemistry (IHC) was performed on 3-μm-thick tissue sections prepared from formalin-fixed, paraffin-embedded tissue from the constructed tissue microarray block. Immunohistochemistry for ER, PR, HER2, EGFR, CK5, P-cadherin, vimentin, and Ki-67 was conducted in accordance to the techniques, antibodies specifications and assay conditions as previously published [30, 31].

The expression of ERCC1 was evaluated using a mouse monoclonal antibody (clone 8F1, Neomarkers, Fremont, California, USA). Sections were deparaffinized with xylene and rehydrated in a series of decreasing concentration of ethanol solutions. For epitope retrieval, sections were exposed to EDTA buffer (pH 9.0) and heated for 30 minutes in a 98º water bath. A 3% hydrogen peroxidase solution was then used to block endogenous peroxidase. Slides were then incubated with the monoclonal antibody at a 1:100 dilution and were labeled with the Envision Detection System from DAKO. DAB plus (3,3'-diaminobenzidine tetrahydrochloride, DAKO Glostrup, Denmark) was then applied as chromogenic substrate and hematoxylin/ammoniacal water as counterstaining. Sections from normal human tonsil tissue were used as an external positive control. For the negative control, the primary antibody was replaced with PBS/nonimmune mouse serum.

A pathologist (RG), who was blinded to the patients' outcomes, assessed the semi-quantitative expression of ERCC1. The scoring system used was previously described by Al Haddad et al. (1999) [32] as H score. Nuclear staining intensity of ERCC1 protein was graded on a scale from 0 to 3, with a larger number indicating a higher intensity. The extension of staining was categorized as: 0 = no tumor nuclei expression; 0.1 = 1 to 9% of positive tumor nuclei; 0.5 = 10 to 49% of positive tumor nuclei; and 1.0 = 50% or more positive tumor nuclei. The extension score was multiplied by the staining intensity of nuclei to obtain a final semi-quantitative H score. The cut-off established for separating ERCC1-positive tumors from ERCC1-negative tumors was the median value of all the H scores. Cores with more than 50% of tissue loss or lack of tumor cells were considered not interpretable.

Statistical analysis

Descriptive statistics comparing ERCC1 expression with the clinicopathologic characteristics were analyzed by the chi-square test or, when necessary, by Fisher's exact test. Survival curves were calculated by the Kaplan-Meier method and the differences were assessed by the log-rank test. 60 months was the maximum cut-off value considered, as it is the expected clinical time for breast cancer recurrence. A computer program package StataTM (Version 9.2, StataCorp, College Station, TX, USA) was used for all statistical testing and management of the database, and a significant level of 5% was considered statistically significant.

Results

From the total of 135 cases enrolled for this study, ERCC1 immunohistochemistry analysis was available for 109 cases. ERCC1 expression was localized to the nucleus of neoplastic cells (Figure 1). The median value of H scores was 0.2. Tumors with an H score $\geq$ 0.2 (i.e., tumors with 10% or more positive nuclei for ERCC1 with an immunostaining intensity score of 1, and/or tumors with 1% or more positive nuclei for

ERCC1 with an immunostaining intensity score of at least 2) were considered ERCC1-positive. Of the 109 cases, 58 (53.2%) were positive for ERCC1.

The expression levels of ERCC1 were compared to clinicopathologic features. A significant association was found between ERCC1 expression and tumor size smaller than 2.0 cm (P = 0.007). The expression of ERCC1 was not significantly related to age (P = 0.154), tumor histological grade (P = 0.400), lymph node status (P = 0.565), TNM stage (P = 0.290) and NPI (P = 0.508). Although there was no statistical correlation between tumor histological type and ERCC1 expression (P = 0.360), 8 of 11 (72.7%) cases of special types of invasive breast carcinoma were positive for ERCC1 compared to 48 of 94 (51.1%) cases of invasive ductal carcinoma (Table 2).

Hormone receptors (ER, PR), HER2, EGFR, CK5, P-cadherin and vimentin status were analyzed. ERCC1 expression was significantly associated with ER positivity (P = 0.040) but not with the remainder biomarkers (Table 3). There was no correlation between the expression of ERCC1 and the molecular subtype of breast cancer (P = 0.226). Nevertheless, 16 of 26 (61.5%) cases of TNBC were ERCC1 negative, while in the non-TNBC group, 34 of 82 (41.5%) cases were negative for ERCC1, including 31 and 3 cases of luminal and HER2-overexpressing subtypes, respectively (Table 3).

The five-year OS rate for all patients enrolled in this study was 69.7% (76 of 109 patients were alive at the end point of the study). There was no statistical correlation between ERCC1 expression and OS: the five-year OS rates were 67.2% (39 of 58 patients were alive) for patients with ERCC1-positive tumors and 72.5% for patients with ERCC1-negative tumors (37 of 51 patients were alive) (P = 0.458). DFS data was available for 84 patients and the five-year DFS rate was 81.0% (68 of 84 patients presented no progression of their disease at the last follow-up). There was no statistical correlation between ERCC1 expression and DFS: the five-year DFS rates at the last follow-up were 75.0% (27 of 36 patients without progression of their disease) for patients with ERCC1-positive tumors and 85.4% for patients

with ERCC1-negative tumors (41 of 48 patients without progression of their disease).

Discussion

In the present study, we analyzed the immunohistochemical expression of ERCC1 in a series of patients with primary breast cancer. We showed an association between ERCC1-positive expression and tumor size smaller than 2.0 cm (P = 0.007), but no correlation was found with other clinicopathologic features. Although not statistically significant, the majority (72.7%) of special histological types of invasive breast carcinomas was positive for ERCC1 compared to invasive ductal carcinomas, which were ERCC1-positive in 51.1% of the cases. Tumors with ERCC1 expression were also associated with positivity for ER (P = 0.040), but there was no correlation between the expression of ERCC1 and the other biomarkers studied. We did not find a statistically significant association between ERCC1 expression and the molecular subtypes of breast cancer, but TNBC were more frequently negative for ERCC1 (61.5% of the cases) compared to the non-TNBC, which were negative for this protein in 41.5% of the cases.

The expression of ERCC1 in breast cancer was analyzed in few studies. In a series of 504 women with early stage breast cancer treated with breast conserving surgery and breast irradiation, Goyal et al. (2010) [26] showed that an increased expression of ERCC1 was associated with features related to a better prognosis, including age > 50 years-old, lower T stage, nodal negativity, ER-positivity, and non-triple negative status. We found similar results regarding ERCC1-positive expression with smaller tumor size and ER-positivity. Our study and others showed that ERCC1 expression is lower in TNBC when compared to non-TNBC. Sidoni et al. (2008) [24] analyzed ERCC1 expression in 81 TNBC and found that around one third (32.0%) was positive for this protein. Another study with 230 breast cancer patients revealed a statistically significant correlation between ERCC1 expression and the molecular subtypes of breast cancer (P = 0.013): ERCC1 positivity was higher in luminal A subtype (69.7%) and lower in TNBC

(48.3%) and luminal B (43.5%) subtypes [25]. In general, invasive breast carcinomas classified as TNBC are high-grade ductal carcinomas, with nuclear pleomorphism and high mitotic index [5]. Interestingly, Handra-Luca et al. (2007) [23] showed that head and neck squamous cell carcinomas with lower levels of ERCC1 were histologically less differentiated than tumors with higher levels of this protein.

Chemotherapy composed by platinum drugs result in the formation of platinum-DNA adducts leading to a distortion in the structure of the DNA molecule. These DNA adducts are repaired by enzymes related to the NER pathway, including ERCC1 [9-11]. The integrity of DNA repair systems, specially the NER pathway, is an important predictor of resistance to chemotherapy based on platinum drugs [12]. Wang et al. (2008) [15] studied 46 malignant pleural or peritoneal effusions collected from patients with gastric cancer, gynecological cancer, and NSCLC and evaluated whether the mRNA levels of ERCC1 and breast cancer susceptibility gene 1 (BRCA1) of the collected samples were associated with *in vitro* chemosensitivity to cisplatin and/or docetaxel. The authors showed that for patients with NSCLC, higher mRNA levels of ERCC1 and BRCA1 in the pleural effusions were negatively correlated to chemosensitivity to cisplatin [15].

Currently, a variety of cancers are treated with platinum-based chemotherapy and the enzyme ERCC1 has been postulated as a possible useful predictive and/or prognostic biomarker to this kind of therapy. According to Olaussen et al. (2006) [19], the status of ERCC1 is a determinant factor for the sensitivity of NSCLC to platinum-based chemotherapy. The authors analyzed two groups of patients with completely resected NSCLC: the adjuvant chemotherapy group received cisplatin-based chemotherapy and the control group was only observed. Patients with ERCC1-negative tumors in the adjuvant chemotherapy group had a statistically significant better OS and DFS when compared to the control group; in contrast, there was no significant difference in survival between the two groups in patients with ERCC1-positive tumors [19]. In another study, the negativity for ERCC1 in patients with locally advanced NSCLC treated with platinum-based neoadjuvant concurrent chemoradiotherapy was

associated with a better survival compared to patients whose tumors were ERCC1-positive [20]. Similar results were found for patients treated with platinum regimens for advanced cancers, including gastric [16], colorectal [17, 18], urinary tract [21, 22], and head and neck cancers [23].

Our study and those of Goyal et al. (2010) [26] and Kim et al. (2011) [25] did not find an association between ERCC1 expression and survival for patients with breast cancer. Other studies analyzed the expression of ERCC1 in patients with advanced breast cancer treated with platinum-based regimens. Shao et al. (2009) [27] studied 54 patients with locally advanced or metastatic breast cancer treated with paclitaxel and cisplatin and found that, in multivariate analysis, ERCC1-positivity was associated with a shorter progression free survival (PFS). A more recent study involving 107 breast cancer patients treated with neoadjuvant chemotherapy composed of paclitaxel plus carboplatin showed that ERCC1-negative tumors were related with a higher pathological complete remission (pCR) than tumors positive for ERCC1. [28] In this study, the association of clinicopathologic variables with negativity for ERCC1, beta-tubulin III, and Bcl-2 was a stronger predictive factor for pCR compared to clinicopathologic variables alone or associated with molecular classification of breast cancer [28].

In general, our results and those from Goyal et al. (2010) [26] and Kim et al. (2011) [25] suggest that ERCC1 expression is associated with more favorable clinicopathologic features in patients with breast cancer, including an association with ER expression and luminal subtype. Some studies have shown that breast cancer patients whose tumors are ER-positive have a significantly lower pCR following neoadjuvant chemotherapy when compared to patients with tumors negative for ER [33, 34]. In the study of Chen et al. (2011) [28], patients with ER and PR negative tumors had a significantly higher pCR rate than those with tumors positive for hormonal receptors; and, among the molecular subtypes of breast cancer, luminal A tumors had the lowest pCR rate. Based on this, the 12th St Gallen International Expert Consensus on the Primary Therapy of Early Breast Cancer 2011 advocates that chemotherapy is less useful in patients with breast tumors classified as luminal A subtype, because this subtype is less

responsive to chemotherapy [4]. Our results and those from other studies suggest that the luminal subtype "resistance" to chemotherapy may be related, at least in part, to the integrity of the DNA repair pathways in this subtype, including the NER pathway [25, 26, 28].

In conclusion, the immunohistochemical expression of ERCC1 in our series of breast carcinomas correlated significantly with some favorable prognostic factors such as smaller tumor size and ER-positivity. In contrast to invasive ductal carcinomas, the majority of special histological types of invasive breast carcinomas are positive for ERCC1. Finally, the expression of this protein is lower in TNBC compared to the non-TNBC. Further investigation of ERCC1 expression in a larger population of advanced breast cancer patients treated with chemotherapy platinum-based regimens is warranted to help elucidate its possible role as a predictive and/or prognostic marker, as far as treatment response and survival are concerned.

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References

1. Perou CM, Sorlie T, Eisen MB, van de Rijn M, Jeffrey SS, Rees CA, Pollack JR, Ross DT, Johnsen H, Akslen LA, Fluge O, Pergamenschikov A, Williams C, Zhu SX, Lonning PE, Borresen-Dale AL, Brown PO, Botstein D (2000) Molecular portraits of human breast tumours. Nature 406(6797):747-752. doi:10.1038/35021093

2. Sorlie T, Perou CM, Tibshirani R, Aas T, Geisler S, Johnsen H, Hastie T, Eisen MB, van de Rijn M, Jeffrey SS, Thorsen T, Quist H, Matese JC, Brown PO, Botstein D, Eystein Lonning P, Borresen-Dale AL (2001) Gene expression patterns of breast carcinomas distinguish tumor subclasses with clinical implications. Proc Natl Acad Sci U S A 98(19):10869-10874. doi:10.1073/pnas.191367098

3. Weigelt B, Horlings HM, Kreike B, Hayes MM, Hauptmann M, Wessels LF, de Jong D, Van de Vijver MJ, Van't Veer LJ, Peterse JL (2008) Refinement of breast cancer classification by molecular characterization of histological special types. J Pathol 216(2):141-150. doi:10.1002/path.2407

4. Goldhirsch A, Wood WC, Coates AS, Gelber RD, Thurlimann B, Senn HJ (2011) Strategies for subtypes--dealing with the diversity of breast cancer: highlights of the St. Gallen International Expert Consensus on the Primary Therapy of Early Breast Cancer 2011. Ann Oncol 22(8):1736-1747. doi:10.1093/annonc/mdr304

5. Badve S, Dabbs DJ, Schnitt SJ, Baehner FL, Decker T, Eusebi V, Fox SB, Ichihara S, Jacquemier J, Lakhani SR, Palacios J, Rakha EA, Richardson AL, Schmitt FC, Tan PH, Tse GM, Weigelt B, Ellis IO, Reis-Filho JS (2011) Basal-like and triple-negative breast cancers: a critical review with an emphasis on the implications for pathologists and oncologists. Mod Pathol 24(2):157-167. doi:10.1038/modpathol.2010.200

6. Higgins MJ, Baselga J (2011) Targeted therapies for breast cancer. J Clin Invest 121(10):3797-3803. doi:10.1172/JCI57152

7. Silver DP, Richardson AL, Eklund AC, Wang ZC, Szallasi Z, Li Q, Juul N, Leong CO, Calogrias D, Buraimoh A, Fatima A, Gelman RS, Ryan PD, Tung NM, De Nicolo A, Ganesan S, Miron A, Colin C, Sgroi DC, Ellisen LW, Winer EP, Garber JE (2010) Efficacy of neoadjuvant Cisplatin in triple-

negative breast cancer. J Clin Oncol 28(7):1145-1153. doi:10.1200/JCO.2009.22.4725

8. Hudis CA, Gianni L (2011) Triple-negative breast cancer: an unmet medical need. Oncologist 16 Suppl 1(1-11. doi:10.1634/theoncologist.2011-S1-01

9. Kelland L (2007) The resurgence of platinum-based cancer chemotherapy. Nat Rev Cancer 7(8):573-584. doi:10.1038/nrc2167

10. Martin LP, Hamilton TC, Schilder RJ (2008) Platinum resistance: the role of DNA repair pathways. Clin Cancer Res 14(5):1291-1295. doi:10.1158/1078-0432.CCR-07-2238

11. Vilmar A, Sorensen JB (2009) Excision repair cross-complementation group 1 (ERCC1) in platinum-based treatment of non-small cell lung cancer with special emphasis on carboplatin: a review of current literature. Lung Cancer 64(2):131-139. doi:10.1016/j.lungcan.2008.08.006

12. Reed E (2005) ERCC1 and clinical resistance to platinum-based therapy. Clin Cancer Res 11(17):6100-6102. doi:10.1158/1078-0432.CCR-05-1083

13. Britten RA, Liu D, Tessier A, Hutchison MJ, Murray D (2000) ERCC1 expression as a molecular marker of cisplatin resistance in human cervical tumor cells. Int J Cancer 89(5):453-457

14. Welsh C, Day R, McGurk C, Masters JR, Wood RD, Koberle B (2004) Reduced levels of XPA, ERCC1 and XPF DNA repair proteins in testis tumor cell lines. Int J Cancer 110(3):352-361. doi:10.1002/ijc.20134

15. Wang L, Wei J, Qian X, Yin H, Zhao Y, Yu L, Wang T, Liu B (2008) ERCC1 and BRCA1 mRNA expression levels in metastatic malignant effusions is associated with chemosensitivity to cisplatin and/or docetaxel. BMC Cancer 8(97. doi:10.1186/1471-2407-8-97

16. Kwon HC, Roh MS, Oh SY, Kim SH, Kim MC, Kim JS, Kim HJ (2007) Prognostic value of expression of ERCC1, thymidylate synthase, and glutathione S-transferase P1 for 5-fluorouracil/oxaliplatin chemotherapy in advanced gastric cancer. Ann Oncol 18(3):504-509. doi:10.1093/annonc/mdl430

17. Shirota Y, Stoehlmacher J, Brabender J, Xiong YP, Uetake H, Danenberg KD, Groshen S, Tsao-Wei DD, Danenberg PV, Lenz HJ (2001) ERCC1 and

thymidylate synthase mRNA levels predict survival for colorectal cancer patients receiving combination oxaliplatin and fluorouracil chemotherapy. J Clin Oncol 19(23):4298-4304

18. Kim SH, Kwon HC, Oh SY, Lee DM, Lee S, Lee JH, Roh MS, Kim DC, Park KJ, Choi HJ, Kim HJ (2009) Prognostic value of ERCC1, thymidylate synthase, and glutathione S-transferase pi for 5-FU/oxaliplatin chemotherapy in advanced colorectal cancer. Am J Clin Oncol 32(1):38-43. doi:10.1097/COC.0b013e31817be58e

19. Olaussen KA, Dunant A, Fouret P, Brambilla E, Andre F, Haddad V, Taranchon E, Filipits M, Pirker R, Popper HH, Stahel R, Sabatier L, Pignon JP, Tursz T, Le Chevalier T, Soria JC (2006) DNA repair by ERCC1 in non-small-cell lung cancer and cisplatin-based adjuvant chemotherapy. N Engl J Med 355(10):983-991. doi:10.1056/NEJMoa060570

20. Hwang IG, Ahn MJ, Park BB, Ahn YC, Han J, Lee S, Kim J, Shim YM, Ahn JS, Park K (2008) ERCC1 expression as a prognostic marker in N2(+) nonsmall-cell lung cancer patients treated with platinum-based neoadjuvant concurrent chemoradiotherapy. Cancer 113(6):1379-1386. doi:10.1002/cncr.23693

21. Bellmunt J, Paz-Ares L, Cuello M, Cecere FL, Albiol S, Guillem V, Gallardo E, Carles J, Mendez P, de la Cruz JJ, Taron M, Rosell R, Baselga J (2007) Gene expression of ERCC1 as a novel prognostic marker in advanced bladder cancer patients receiving cisplatin-based chemotherapy. Ann Oncol 18(3):522-528. doi:10.1093/annonc/mdl435

22. Kim KH, Do IG, Kim HS, Chang MH, Kim HS, Jun HJ, Uhm J, Yi SY, Lim do H, Ji SH, Park MJ, Lee J, Park SH, Kwon GY, Lim HY (2010) Excision repair cross-complementation group 1 (ERCC1) expression in advanced urothelial carcinoma patients receiving cisplatin-based chemotherapy. APMIS 118(12):941-948. doi:10.1111/j.1600-0463.2010.02648.x

23. Handra-Luca A, Hernandez J, Mountzios G, Taranchon E, Lacau-St-Guily J, Soria JC, Fouret P (2007) Excision repair cross complementation group 1 immunohistochemical expression predicts objective response and cancer-specific survival in patients treated by Cisplatin-based induction

chemotherapy for locally advanced head and neck squamous cell carcinoma. Clin Cancer Res 13(13):3855-3859. doi:10.1158/1078-0432.CCR-07-0252

24. Sidoni A, Cartaginese F, Colozza M, Gori S, Crino L (2008) ERCC1 expression in triple negative breast carcinoma: the paradox revisited. Breast Cancer Res Treat 111(3):569-570. doi:10.1007/s10549-007-9804-4

25. Kim D, Jung W, Koo JS (2011) The expression of ERCC1, RRM1, and BRCA1 in breast cancer according to the immunohistochemical phenotypes. J Korean Med Sci 26(3):352-359. doi:10.3346/jkms.2011.26.3.352

26. Goyal S, Parikh RR, Green C, Schiff D, Moran MS, Yang Q, Haffty BG (2010) Clinicopathologic significance of excision repair cross-complementation 1 expression in patients treated with breast-conserving surgery and radiation therapy. Int J Radiat Oncol Biol Phys 76(3):679-684. doi:10.1016/j.ijrobp.2009.02.050

27. Shao YY, Kuo KT, Hu FC, Lu YS, Huang CS, Liau JY, Lee WC, Hsu C, Kuo WH, Chang KJ, Lin CH, Cheng AL (2010) Predictive and prognostic values of tau and ERCC1 in advanced breast cancer patients treated with paclitaxel and cisplatin. Jpn J Clin Oncol 40(4):286-293. doi:10.1093/jjco/hyp184

28. Chen X, Wu J, Lu H, Huang O, Shen K (2012) Measuring beta-tubulin III, Bcl-2, and ERCC1 improves pathological complete remission predictive accuracy in breast cancer. Cancer Sci 103(2):262-268. doi:10.1111/j.1349-7006.2011.02135.x

29. Matos I, Dufloth R, Alvarenga M, Zeferino LC, Schmitt F (2005) p63, cytokeratin 5, and P-cadherin: three molecular markers to distinguish basal phenotype in breast carcinomas. Virchows Arch 447(4):688-694. doi:10.1007/s00428-005-0010-7

30. Dufloth RM, Matos I, Schmitt F, Zeferino LC (2007) Tissue microarrays for testing basal biomarkers in familial breast cancer cases. Sao Paulo Med J 125(4):226-230

31. Sousa B, Paredes J, Milanezi F, Lopes N, Martins D, Dufloth R, Vieira D, Albergaria A, Veronese L, Carneiro V, Carvalho S, Costa JL, Zeferino L, Schmitt F (2010) P-cadherin, vimentin and CK14 for identification of basal-

like phenotype in breast carcinomas: an immunohistochemical study. Histol Histopathol 25(8):963-974

32. Al-Haddad S, Zhang Z, Leygue E, Snell L, Huang A, Niu Y, Hiller-Hitchcock T, Hole K, Murphy LC, Watson PH (1999) Psoriasin (S100A7) expression and invasive breast cancer. Am J Pathol 155(6):2057-2066. doi:10.1016/S0002-9440(10)65524-1

33. Ring AE, Smith IE, Ashley S, Fulford LG, Lakhani SR (2004) Oestrogen receptor status, pathological complete response and prognosis in patients receiving neoadjuvant chemotherapy for early breast cancer. Br J Cancer 91(12):2012-2017. doi:10.1038/sj.bjc.6602235

34. Colleoni M, Bagnardi V, Rotmensz N, Gelber RD, Viale G, Pruneri G, Veronesi P, Torrisi R, Cardillo A, Montagna E, Campagnoli E, Luini A, Intra M, Galimberti V, Scarano E, Peruzzotti G, Goldhirsch A (2009) Increasing steroid hormone receptors expression defines breast cancer subtypes non responsive to preoperative chemotherapy. Breast Cancer Res Treat 116(2):359-369. doi:10.1007/s10549-008-0223-y

Table 1 Clinicopathological characteristics of the 109 patients for whom ERCC1 immunochemistry was available

Feature	No.	%
Age (years)		
< 50	26	23.9
≥ 50	83	76.1
Histological type		
IDC	94	86.2
ILC	3	2.8
IC – Mixed	1	0.9
IC – Special type	11	10.1
Histological grade		
I	16	14.7
II	52	47.7
III	41	37.6
Tumor size		
< 2.0 cm	41	39.4
≥ 2.0 cm	63	60.6
Lymph node status		
Negative	56	53.8
Positive	48	46.2
ER		
Negative	34	31.5
Positive	74	68.5
PR		
Negative	58	53.7
Positive	50	46.3
HER2 status		
Negative	100	92.6
Positive	8	7.4
EGFR		
Negative	61	92.4
Positive	5	7.6

Table 1 (continued)

CK5		
Negative	77	71.3
Positive	31	28.7
P-cadherin		
Negative	74	67.9
Positive	35	32.1
Vimentin		
Negative	43	82.7
Positive	9	17.3
Molecular subtype		
Luminal	75	69.4
HER2-overexpressing	7	6.5
TNBC	26	24.1
Ki-67		
Low proliferative	37	56.1
High proliferative	29	43.9
ERCC1		
Negative	51	46.8
Positive	58	53.2
TNM stage		
I	30	28.8
II	40	38.5
III	10	9.6
IV	24	23.1
NPI		
Good prognosis	20	21.3
Moderate prognosis	53	56.4
Poor prognosis	21	22.3

CK5 cytokeratin 5, *EGFR* epidermal growth factor receptor, *ER* estrogen receptor, *ERCC1* excision repair cross-complementation 1 enzyme, *HER2* human epidermal growth factor receptor 2, *IC* invasive carcinoma, *IDC* invasive ductal carcinoma, *ILC* invasive lobular carcinoma, *NPI* Nottingham Prognostic index, *PR* progesterone receptor, *TNBC* triple negative breast cancer

Table 2 Clinicopathological characteristics of the patients according to ERCC1 status

	ERCC1 negative H score < 0.2 (*n* = 51)	**ERCC1 positive H score ≥ 0.2 (*n* = 58)**	***P* value**
Age (years)			
< 50	9 (34.6)	17 (65.4)	0.154
≥ 50	42 (50.6)	41 (49.4)	
Histological type			
IDC	46 (48.9)	48 (51.1)	0.360
ILC	1 (33.3)	2 (66.7)	
IC – Mixed	1 (100.0)	0 (0.0)	
IC – Special type	3 (27.3)	8 (72.7)	
Histological grade			
I	5 (31.3)	11 (68.8)	0.400
II	26 (50.0)	26 (50.0)	
III	20 (48.8)	21 (51.2)	
Tumor size			
< 2.0 cm	13 (31.7)	28 (68.3)	0.007*
≥ 2.0 cm	37 (58.7)	26 (41.3)	
Lymph node status			
Negative	26 (46.4)	30 (53.6)	0.565
Positive	25 (52.1)	23 (47.9)	
TNM stage			
I	10 (33.3)	20 (66.7)	0.290
II	22 (55.0)	18 (45.0)	
III	5 (50.0)	5 (50.0)	
IV	13 (54.2)	11 (23.1)	
NPI			
Good prognosis	8 (40.0)	12 (60.0)	0.508
Moderate prognosis	28 (52.8)	25 (47.2)	

Table 2 (continued)

Poor prognosis	12 (57.1)	9 (42.9)

ERCC1 excision repair cross-complementation 1 enzyme, *IC* invasive carcinoma, *IDC* invasive ductal carcinoma, *ILC* invasive lobular carcinoma, *NPI* Nottingham Prognostic índex

* *P* value is statiscally significant

Table 3 Biomarkers expression according to ERCC1 status

	ERCC1 negative H score < 0.2 (*n* = 51)	**ERCC1 positive H score ≥ 0.2 (*n* = 58)**	***P* value**
ER			
Negative	21 (61.8)	13 (38.2)	0.040*
Positive	30 (40.5)	44 (59.5)	
PR			
Negative	27 (46.6)	31 (53.4)	0.881
Positive	24 (48.0)	26 (52.0)	
HER2			
Negative	47 (47.0)	53 (53.0)	0.722
Positive	3 (37.5)	5 (62.5)	
EGFR			
Negative	39 (63.9)	22 (36.1)	0.651
Positive	4 (80.0)	1 (20.0)	
CK5			
Negative	39 (50.6)	38 (49.4)	0.153
Positive	11 (35.5)	20 (64.5)	
P-cadherin			
Negative	34 (45.9)	40 (54.1)	0.798
Positive	17 (48.6)	18 (51.4)	
Vimentin			
Negative	24 (55.8)	19 (44.2)	0.283
Positive	7 (77.8)	2 (22.2)	
Molecular subtype			
Luminal	31 (41.3)	44 (58.7)	0.226
HER2-overexpressing	3 (42.9)	4 (57.1)	
TNBC	16 (61.5)	10 (38.5)	
Ki-67			
Low proliferative	25 (67.6)	12 (32.4)	0.642

Table 3 (continued)

High proliferative	18 (62.1)	11 (37.9)

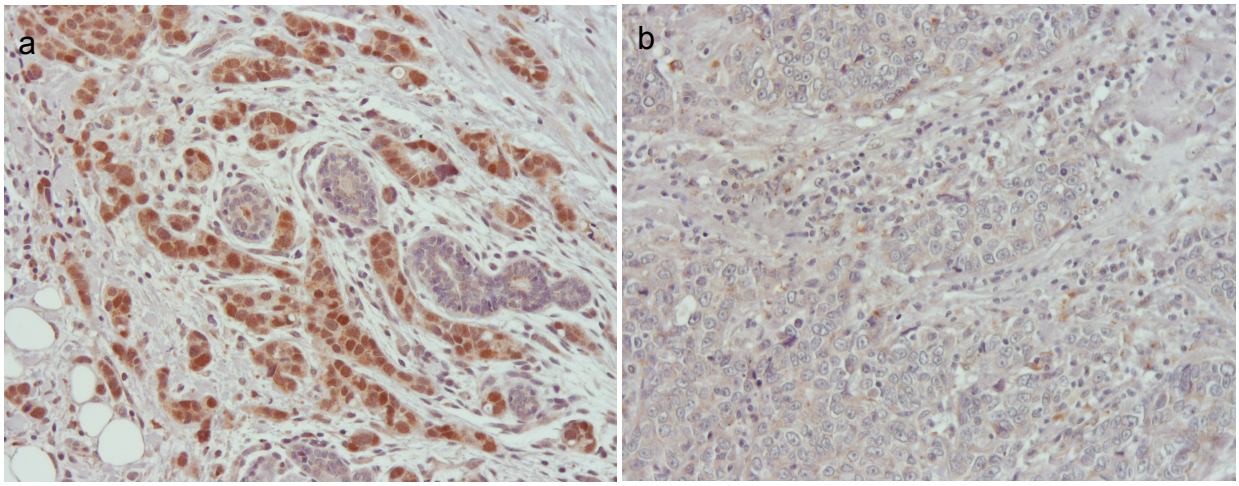
CK5 cytokeratin 5, *EGFR* epidermal growth factor receptor, *ER* estrogen receptor, *ERCC1* excision repair cross-complementation 1 enzyme, *HER2* human epidermal growth factor receptor 2, *PR* progesterone receptor, *TNBC* triple negative breast cancer

* *P* value is statiscally significant

Figure Captions

Fig. 1 Representative immunohistochemical staining for ERCC1 in breast cancer. **a** Diffuse expression for ERCC1 in the nuclei of breast tumor cells with intensity staining scored as 3 (H score = 3) (original magnification, x200). **b** Breast tumor tissue negative for ERCC1 expression (H score = 0) (original magnification, x200)

Fig. 1



ANEXOS

Anexo 1: Normas de Publicação da Revista "Virchows Archiv"

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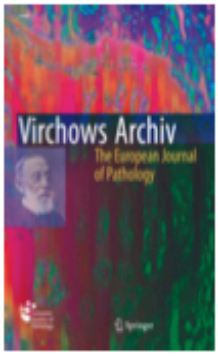

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Instructions for Authors

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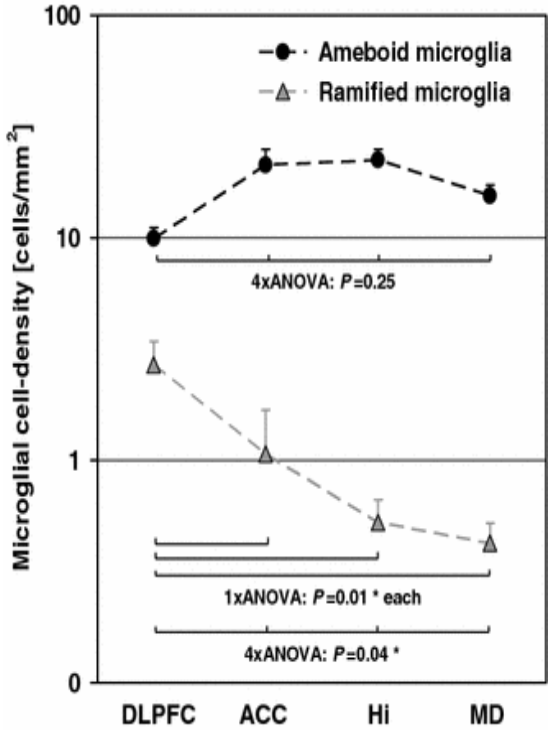

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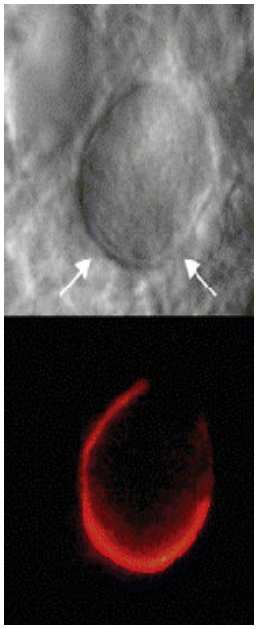

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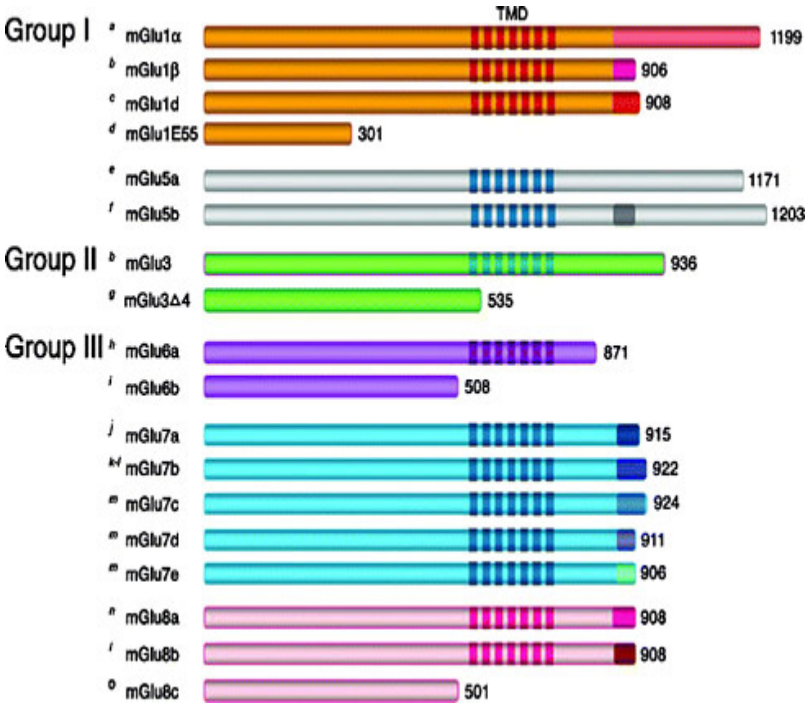

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