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AGR2 associates with HER2 expression predicting poor outcome in subset of estrogen receptor negative breast cancer patients

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ABSTRACT

Expression of the AGR2 oncogene was shown to be associated with estrogen receptor positive tumors. This gene contributes to enhanced cellular proliferation, drug resistance, metastasis development and may also serve as a predictor of poor prognosis. However, our analysis of AGR2 expression in a subset of estrogen-receptor negative tumors revealed that AGR2 could also be upregulated in hormone-independent manner. AGR2 expression was shown to be significantly increased in HER2 positive breast tumors on both the mRNA and the protein level. Moreover, in a subset of estrogen- and progesterone-receptor negative and simultaneously HER2-positive cases, increased AGR2 expression significantly correlated with worse patient prognosis. Subsequent analysis of independent data sets either collected in our institute or obtained from Oncomine cancer microarray database confirmed all these findings.

1. Introduction

Breast cancer is the most frequently diagnosed female cancer in the world with an estimated 1.7 million cases and 521,900 deaths in 2012. This malignancy alone accounts for 25% of all cancer cases and is responsible for 15% of all cancer-related deaths in women (Torre et al., 2015). The progress in molecular biology and genomics in the past decade has had significant impact on raising the efficiency of diagnostics and treatment of different types of malignancies, including breast cancer. It should be noted that breast cancer is a group of heterogeneous diseases; however, subsets of these tumors show recurrent patterns of transcriptional, genomic, and biological abnormality. Gene expression profiling has identified several major breast cancer subtypes contributing to prediction of disease outcome (van 't Veer et al., 2002). The most reproducibly identified molecular subtypes are the luminal A, luminal B, HER2 and basal-like groups (Schnitt, 2010). On the other hand, the three biomarkers that are routinely used to assess breast tumors in clinical practice - estrogen receptor (EsR), progesterone receptor (PR) and human epidermal growth factor receptor (ERBB2 or HER2) can be used to approximate the molecular category of breast cancer, as defined by gene expression profiling (Brenton et al., 2005). These three gene products play an important role in driving breast cancer development and anti-estrogen or anti-HER2 therapies are proving to be useful in treating many breast tumors expressing these receptors.

Approximately 70% of all breast tumors express EsR, serving as a positive prognostic marker and a predictive marker of response to endocrine therapy (Stanford et al., 1986). In the late 1990's, co-expression of the AGR2 (anterior gradient-2) gene and EsR was detected in mammary gland derived cancer cells (Thompson and Weigel, 1998). Later, it was shown that AGR2 expression is directly stimulated by EsR signaling (Hrstka et al., 2010; Salmans et al., 2013) and correlates with poor outcome in patients with breast cancer (Hrstka et al., 2015; Hrstka et al., 2013a; Innes et al., 2006). Aberrant AGR2 expression was then detected also in other human malignancies, both hormone-dependent such as breast, ovarian and prostate cancer and hormone-independent including that of gastro-intestinal tract, oral cavity, lungs and esophagus (Brychtova et al., 2011). Many studies *in vitro* and *in vivo* show that AGR2 contributes to cancer cell proliferation and survival, tumor growth and invasion and metastasis formation (Di Maro et al., 2014; Wang et al., 2008), but the exact mechanisms for its oncogenic activities are not fully understood. AGR2 has the structural features of a protein disulfide isomerase and therefore is likely to be important for folding of other proteins involved in cellular response to stress (Higa et al., 2011). As a secreted protein, it can also interact with extracellular domains of membrane proteins, modulating their activity and/or affecting associated intracellular pathways (Dumartin et al., 2011; Ramachandran et al., 2008).

Estrogen independent regulation of AGR2 expression in breast tumors was already indicated in our previous work showing modulation

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of AGR2 level via PDK-1/AKT signaling pathway as well as the rare detection of AGR2 by immunohistochemical staining (IHC) in EsR- and PR-negative specimens (Hrstka et al., 2013b). In this work we aimed to identify specific molecular pattern indicating AGR2 expression in EsR/PR-negative breast tumors and to analyze the consequences of elevated AGR2 levels for patient outcomes.

2. Material and methods

2.1. Clinical samples and processing

The first cohort consisted of 211 tumor core biopsies collected from consecutive patients with EsR- and PR-negative invasive primary breast carcinoma treated at Masaryk Memorial Cancer Institute during 2004–2009. Material from 149 of these specimens was also stored in our Tissue Biobank in RNAlater solution (Sigma-Aldrich, St. Louis, MO, USA). These samples were subjected to RT-qPCR analysis.

The second cohort of 96 primary breast carcinomas with protein and transcript data available was used to confirm data from the first cohort. Of these, 7 EsR −/PR −/HER2 + and 16 EsR −/PR −/HER2 − patients, all grade 3 tumors, were analyzed as previously described using RT-qPCR (Bouchal et al., 2015), targeted proteomics (Prochazkova et al., 2017) and (Table S1) and immunohistochemistry (see below).

All specimens were received within 20 min of surgical removal according to standardized hospital protocol and immediately evaluated by a pathologist. Tissue blocks were fixed in 4% neutral formaldehyde for approximately 24 h before processing into paraffin wax.

All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards. Informed consent was obtained from all individual participants included in the study.

2.2. Reverse transcription and quantitative PCR

cDNA synthesis was carried out using M-MLV reverse transcriptase (Invitrogen, Carlsbad, CA, USA). Triplicate samples were subjected to quantitative PCR analysis using SYBR Green for AGR2 and β-actin as an endogenous control and TaqMan for 18S rRNA (Applied Biosystems, Foster City, CA, USA) that was used as an additional endogenous control. Primer pairs are the same as described previously (Hrstka et al., 2010).

2.3. Immunohistochemistry and analysis of staining patterns

Immunohistochemical staining was performed on 4 μm freshly cut tissue sections. Sections were deparaffinized in xylene and rehydrated into PBS through a graded ethanol series. Endogenous peroxidase activity was quenched in 3% hydrogen peroxide in PBS for 15 min. Antigen retrieval was performed in citrate buffer pH 6 at 94 °C for 20 min. The sections were incubated overnight at 4 °C with anti-AGR2 antibody (polyclonal, Sigma-Aldrich, St. Louis, MO, USA), MUC1 (Clone Ma 695, Leica Biosystems, Newcastle Upon Tyne, UK) and MUC4 (Clone 1G8, Santa Cruz Biotechnology, Dallas, Texas, USA). Tumor cells were classified into four staining intensities: 0–3 (0 no staining, 1 weak staining, 2 positive staining, 3, strong staining). The percentages of cells in each category were multiplied by the intensity value and the products added to each other to obtain the Histoscore (Krekac et al., 2008). By definition, the values ranged from 0 to 300 for each antibody. Histoscores were categorized as negative (0–10), weak (11–50), moderate (51–150) and strong (151–300). HER2 status was determined using HerceptTest (Dako, Glostrup, Denmark) according to recommended scoring system 0–3 +. Cases with staining scored as 0 or 1 + were denoted as HER2– negative. Cases scored as 3 + and 2 + cases with confirmed HER2 amplification by FISH were denoted as HER2–positive. HER2 cases scored as 2 + without confirmed gene amplification were excluded from the study.

2.4. Data mining and statistical methods

Oncomine, a cancer microarray database, was screened for breast cancer datasets where EsR, PR and HER2 status was determined (www.oncomine.com) (Rhodes et al., 2004). The six largest datasets including Curtis (Curtis et al., 2012), TCGA, Bonnefoi (Bonnefoi et al., 2007), Bittner, Kao (Kao et al., 2011) and Gluck (Gluck et al., 2012) were analyzed for AGR2 expression. The overall number of patients in datasets/number of HER2 + EsR-PR-patients/number of triple negative patients were as follows: Curtis: 2136/109/211, TCGA: 593/14/46, Bonnefoi: 160/29/80, Bittner: 336/19/38, Kao: 327/25/32, Gluck: 158/18/50. Positive association between HER2 and elevated AGR2 expression was considered significant at the level of $p < 0.05$ (Mann-Whitney *U* test).

To assess the relevance of HER2 with AGR2 expression, we used the online survival analysis software Kaplan-Meier Plotter (http://kmplot.com/analysis/index.php?p=service) based on data from 4142 breast cancer patients (Gyorffy et al., 2010).

Statistical analysis was performed using STATISTICA 12 (StatSoft, Tulsa, Oklahoma, USA). For comparison of RT-qPCR data as well as IHC and SRM results, the nonparametric Kruskal-Wallis test or Mann-Whitney *U* test were used. These tests were also used in Table 1, unless otherwise stated. One-way analysis of variance (ANOVA) for independent samples was used to test significant differences between the means of analyzed groups. Data for Fig. S1 were generated in R (language for statistical computing version 3.2.2, function wilcox.test, default settings). The associations between AGR2 expression and HER2 status were assessed by Wilcoxon rank sum test for all three data sets obtained from RT-qPCR, SRM and IHC.

3. Results

3.1. AGR2 expression correlates with HER2 intrinsic subtype

AGR2 was detected by IHC in a cohort of 211 consecutive EsR- and PR-negative breast cancer specimens and statistically evaluated in

Table 1
IHC determination of AGR2 protein in relation to clinicopathologic variables.

Variable	Number of all samples	AGR2 (p-level)	AGR2 (Her2 +)[a]
Number of samples	211		66
Histological type			
Ductal	171	0.194	0.341
Lobular	6		
Other	27		
Nodal status			
Negative	91	0.667	0.383
Positive	95		
Histological grade			
G1	1	0.302	0.969
G2	13		
G3	182		
Her2 expression			
0–1	143	<0.001	ND
2–3	66		
Tumor size			
pT1	70	0.863	0.392
pT2	105		
pT3	11		
pT4	14		
MUC1 expression[b]	167	R = 0.225 0.004	R = 0.666 <0.001
MUC4 expression[b]	133	0.248	ND

ND not determined.
[a] Her2 positive only.
[b] Spearman rank correlation.