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ACE and ACE2 Protein Expression Changes with Tumour Grade in Invasive Ductal Carcinomas.

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Renin-angiotensin system is mainly known as a regulator of cardiovascular homeostasis. The aim of the present study was to determine the immunohistochemical expression of angiotensin-converting enzyme and angiotensin-converting enzyme 2 in non-tumorous breast tissue and in G1, G2 and G3 invasive ductal carcinomas, using immunoperoxidase method on formalin fixed paraffin embedded tissue sections from 10 samples of non-tumorous breast tissue and 30 cases of invasive ductal carcinoma. It was found that ACE was located only in ductal epithelium, while in invasive carcinomas, stromal cells were also positive for ACE. Intensity of staining increased with tumour grade. None of the examined invasive carcinomas showed positive staining for ACE2 in tumour epithelial cells, but weak staining was observed in stromal cells adjacent to tumour epithelial cells. In higher grade tumours, less stromal cells were positive for ACE2. These observations suggest that ACE and ACE2 may be involved in the pathogenesis of breast cancer.

Key words: breast cancer, ACE, ACE2

Introduction

The renin-angiotensin system (RAS) consists of systemic and local parts. The systemic RAS has been mainly perceived as an important regulator of cardiovascular homeostasis and as a key factor in the pathogenesis of hypertension and atherosclerosis. The local renin-angiotensin systems observed in various organs act mainly over a limited area. The first element of RAS is liver-derived angiotensinogen, a glycoprotein cleaved by renin to generate decapeptide angiotensin I (Ang I). The angiotensin-converting enzyme (ACE) is a protease capable of cleaving the inactive Ang I to active octapeptide angiotensin II (Ang II), which is regarded as the most active regulator of the systemic RAS [17]. The actions of Ang II are mediated predominantly through its specific receptors, Ang II receptor type 1 (AT1R) and Ang II receptor type 2 (AT2R) [6]. Most of the known effects of Ang II – such as its stimulation of angiogenesis, cellular proliferation, inflammatory and antiapoptotic responses – occur via AT1R [12, 13]. AT2R-mediated actions have been shown to oppose those elicited by AT1R [27, 29]. However, several lines of evidence

suggest that signalling via AT2R may also be proangiogenic and proinflammatory [26, 30]. Although Ang II is the most important effector of the RAS, there are also other products of aminopeptidase activity of ACE and angiotensin converting enzyme 2 (ACE2), such as angiotensin III (Ang III), angiotensin IV (Ang IV), and angiotensin-(1-7) [(Ang-(1-7)], which all show potent biological activity [19, 31, 16, 24]. Ang-(1-7) is an endogenous 7-amino-acid peptide hormone that exerts antiproliferative activity and counteracts the vasodilative and apoptotic properties of Ang II [2]. The specific effects of Ang-(1-7) are mediated by a recently identified receptor, the *mas* oncogene product (MAS) [14]. Ang-(1-7) may be formed from Ang I through cleavage of angiotensin-(1-9), or it may be generated directly from Ang II by the enzymatic activity of ACE2. ACE2, discovered almost a decade ago, is an ACE homologue and a zinc-metallopeptidase. It has been suggested that ACE2 may oppose the effects of ACE on the organ and tissue levels through the generation of Ang-(1-7) by the local RAS. The local RAS systems have been detected in various species and in diverse organs, such as the brain, the testes, the prostate, the pancreas, the adrenal gland, and the mammary gland [7, 11, 15, 21, 22, 25, 28]. The local RAS systems enable the generation of Ang II, and may therefore exert biological activity on an organ level. Recent studies suggest that, on a tissue level, the local RAS may influence cell proliferation and apoptosis, which are considered crucial in carcinogenesis [1, 18].

Invasive ductal carcinoma is the most common type of breast cancer. According to the degree of tubule formation, nucleus pleomorphism and mitotic index, invasive carcinomas are classified as well-differentiated (low-grade) – Grade 1 (G1), moderately differentiated (medium-sized) – Grade 2 (G2) and low-differentiated (high-grade) – Grade 3 (G3). Data on ACE and ACE2 protein expression in breast carcinomas are scarce and in none of these studies tumour grade was taken into account.

The aim of the current study was to investigate ACE and ACE2 protein expression in non-tumorous breast tissue and Grade 1 (G1), Grade 2 (G2) and Grade 3 (G3) invasive ductal carcinomas.

Materials and Methods

Thirty samples of invasive ductal carcinoma (10 cases of highly differentiated (G1) ductal carcinoma, 8 cases of moderately differentiated (G2) ductal carcinoma and 12 cases of low-differentiated (G3) ductal carcinoma) and 10 samples from non-tumorous breast tissue were included in the study.

Tissue samples were fixed in 10% buffered formalin, dehydrated and embedded in paraffin. Paraffin sections, 5 μm thick, were stained with hematoxylin and eosin for histopathological evaluation.

Immunohistochemistry was performed on paraffin embedded 5 μm tissue sections, following antigen retrieval in Citrate Buffer, pH 6.0 (ScyTek Laboratories Inc.,USA) at 95°C for 20 min. Endogenous peroxidase activity was blocked with 3% H_2O_2 for 10 min at room temperature. Subsequently, the sections were washed in TTBS (tris-buffered saline + 0,05% Tween 20) and incubated with primary antibodies against ACE (1:500, rabbit monoclonal, Abcam), ACE2 (1:150, Rabbit monoclonal, Abcam). Biotin-Streptavidin HRP detection system (ScyTek Laboratories Inc., USA) with DAB as chromogen was used.

Results

Immunohistochemical examination in non-tumorous tissue showed weak staining for ACE located only in ductal epithelial (**Fig. 1 a**). In invasive carcinomas, tumour epithelial cells were also stained, but intensity of staining increased in higher grade carcinomas (Fig. 1 c, d) with the most intense staining being observed in G3. While reaction for ACE was absent in stromal cells of non-tumourous tissue, in G1 invasive carcinomas staining reaction was observed in single stromal cells (**Fig. 1 b**) and in G2 and G3 invasive carcinomas there was intensive staining of stromal cells (**Fig. 1 c, d**).

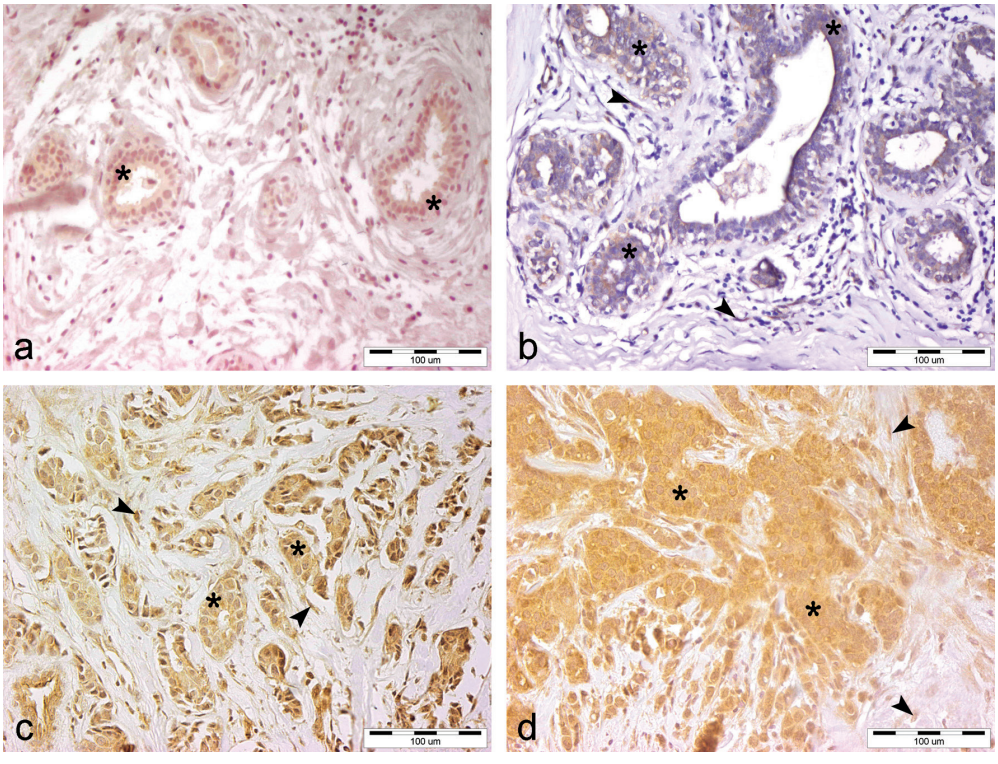

Fig. 1. Immunohistochemical localization of ACE in a) non-tumourous breast tissue; b) invasive highly differentiated (G1) ductal carcinoma; c) invasive moderately differentiated (G2) ductal carcinoma and d) invasive low differentiated (G3) ductal carcinoma. Positive staining for ACE in epithelial cells (asterisk) and stromal cells (arrowhead).

In non-tumourous breast tissue, ACE2 was predominantly located in ductal epithelium, showing intense apical staining and weaker cytoplasmic reaction, as well as in some of the stromal cells (**Fig. 2 a**). None of the examined invasive carcinomas showed positive staining for ACE2 in tumour epithelial cells, but weak staining was observed in stromal cells adjacent to tumour epithelial cells (**Fig. 2 b, c, d**). In higher grade tumours less stromal cells were positive.

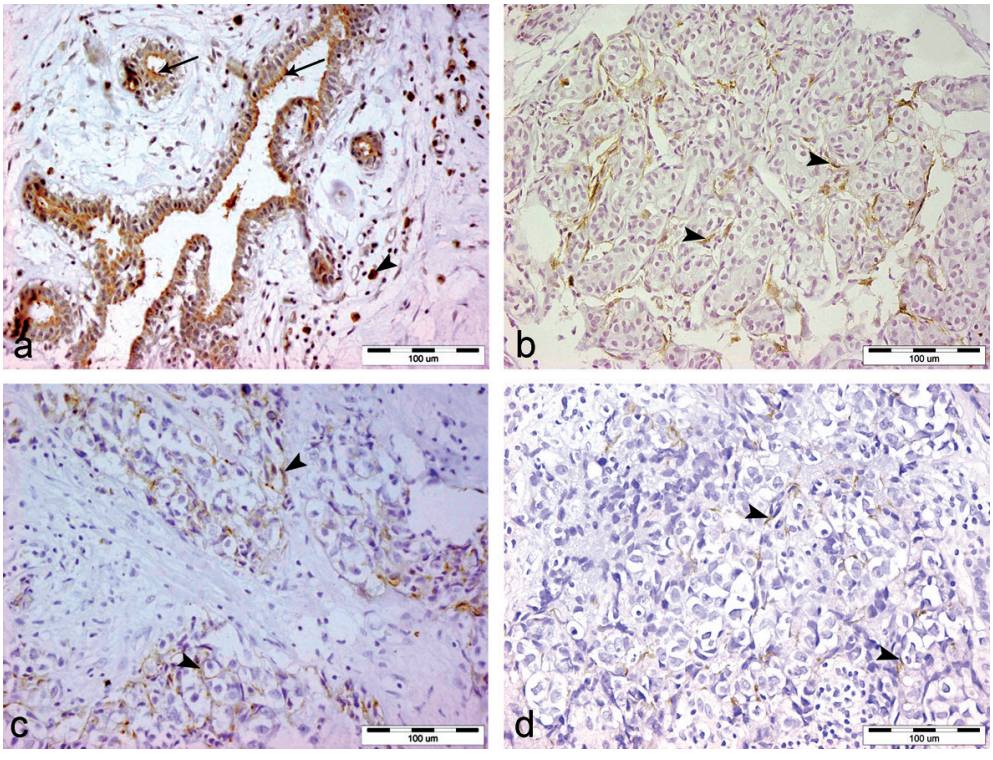

Fig. 2. Immunohistochemical localization of ACE2 in a) non-tumourous breast tissue; b) invasive highly differentiated (G1) ductal carcinoma; c) invasive moderately differentiated (G2) ductal carcinoma and d) invasive low differentiated (G3) ductal carcinoma. Apical reaction in ductal epithelium (arrow) and in stromal cells (arrowhead).

Discussion

Breast cancer is the most common spontaneously diagnosed malignancy and one of the leading causes of death in women. The role of the renin-angiotensin system (RAS) in the development of various malignancies has been extensively investigated in recent years and its impact and involvement in tumour growth, cell proliferation and migration have been demonstrated [5].

Current study showed a different trend in the expression of ACE and ACE2 in different grades of invasive ductal carcinomas. With the decrease in differentiation, ACE protein expression was increased, whereas for ACE2 an inverse relationship was observed. Increased ACE expression has been reported to be associated with enhanced tumour progression and metastasis, including in breast cancer. Similar correlation has been found in other types of tumours. Han C. et al. have reported that using qRT-PCR they had found significant increase in ACE expression in laryngeal cancer, which had been associated with unfavourable prognosis and higher risk of tumour metastases [10]. However, the level of ACE expression in malignant tumour cells does not always correlate with the intensity of Ang II formation due to the active chymase regulating the ACE-independent Ang II formation pathway [32].

Our immunohistochemical analysis of ACE2 showed lack of protein expression in tumour epithelial cells and reduction in the staining of the stromal cells in these tumours

with the increase of their grade. This is logical, since ACE2 is responsible for the conversion of Ang II to Ang-(1-7), which stimulates apoptosis and suppresses cell proliferation, exerting its regulatory effect via the MAS1 receptor. According to some authors, the reduction of ACE2 expression in breast cancer is considered to be a marker of severe disease with a high risk of metastases. Increased expression of the ACE2/Ang-(1-7)/MAS1 axis inhibits cell migration and invasion *in vivo* and *in vitro* in breast cancer, while the reduction in its expression enhances breast cancer metastases by activating PAK1/NF-κB/Snail1 pathways [4,33]. It has been reported that the level of expression of ACE2 negatively correlates with the intensity of neoangiogenesis in non-small cell lung carcinoma. The ACE2/Ang-(1-7)/MAS1 axis inhibits VEGFA secretion, while matrix metalloproteinases MMP-2 and MMP-9, help limit neoangiogenesis, increase tumour sensitivity to cytostatic drugs, and reduce the risk of metastases [3, 9]. Numerous studies have shown that hypoxia is a distinctive feature of solid tumours and that inadequate oxygen supply contributes to the pro-oncogenic effect of ACE/Ang II at the background of reducing the effects of the ACE2/Ang-(1-7)/MAS1 axis [8]. There is enough evidence that support the inclusion of Ang-(1-7) in clinical practice as a treatment option of triple-negative breast cancer [14, 20].

Conclusions

In conclusion, the present study demonstrated a different trend in ACE and ACE2 expression. ACE protein expression was increased in higher grade tumours, whereas the opposite was observed for ACE2 protein expression. These results further support the role of ACE and ACE2 in the progression of invasive ductal carcinomas and may be useful in the development of effective therapies using ACE inhibitors.

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