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RESEARCH ARTICLE

A High Expression of AZIN1 but not AZIN2 is involved in the progression of Breast Cancer

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Abstract

Dormancy of breast cancer after removing the primary tumor is unpredictable, and depends on both the features of the malignant epithelial cells and on the immunological response of the host. In this study we investigated the role of antizyme inhibitors AZIN1 and AZIN2, important regulators of ornithine decarboxylase, in the biological behaviour of breast cancer. Ornithine decarboxylase is the rate-limiting enzyme to produce polyamines from ornithine, crucial for cell proliferation and tumor induction and spread. In the tumor stroma polyamines attenuate the immunological response of the micro environment towards malignant cells. We used 137 paraffin fixed samples of primary breast cancer and their corresponding recurrent lesions to evaluate the expression of AZIN1 and AZIN2 at protein level. AZIN1 was expressed at a significantly higher level in recurrences than in the corresponding primary tumors. Moreover, we found a high expression of AZIN1 in tumor cells and in the epithelium of benign glands in the samples of primary tumors to predict an early progression of breast cancer. A high expression of AZIN1 in primary tumors correlated with HER2 gene amplification and progesteron receptor positivity. AZIN2, likely to be involved in cell secretion, did not correlate with the biological behaviour of the tumor.

Keywords: Breast cancer, Dormancy, Antizyme inhibitors, Tumor microenvironment

Introduction

Induction of polyamines has been shown to be essential for neoplastic growth [1-4], and the regulation of their synthesis has a profound impact both on the proliferation of the malignant cells, and on the development of the immunological response to the tumor. Polyamines are produced from the amino acid ornithine in a rate-limiting reaction catalyzed by the enzyme ornithine decarboxylase (ODC), which is regulated by antizymes (AZs), and antizyme inhibitors (AZINs), to maintain the polyamine homeostasis within the cell. All antizyme proteins, AZ1-4, inhibit ODC activity [5]. Antizyme inhibitors AZIN1 and AZIN2 efficiently negate the activities of AZs. AZIN1 is ubiquitously expressed, whereas AZIN2 is abundant in testes and brain, and in various cell types with secretory or vesicle transport activity [6]. Both antizyme inhibitors, AZIN-1 and AZIN-2 interact with all AZ proteins, and inhibit their function as down-regulators of the activity of ODC. AZIN1 is intimately associated with cell cycle control and proliferation, and is emerging as an oncogene, whereas AZIN2 is expressed in terminally differentiated cells and seems to have a role in controlling cell secretion. In this study we investigated by immuno-histochemistry (IHC) the expression of AZIN1 and AZIN2 in a set of samples from 137 primary breast cancers and the corresponding recurrent or metastatic lesions, in order to elucidate the role of the regulation of polyamine metabolism in tumor dormancy and progression, and to understand the putative difference of the impact of AZIN1 versus AZIN2. The entire set of samples was divided into three Groups (1, 2, 3) according to the length of the dormancy between the primary operation and the detection of the recurrent or metastatic lesion (Group 1 for 0-2 years, Group 2 for 5-10 years, Group 3 for over 10 years).

Materials and Methods

Tissue samples

Paraffin-embedded tissue blocks from 137 primary tumors (PTs) and their corresponding recurrent lesions (Rs) were collected from the archives of the Department of Pathology, the University Hospital of Helsinki as described previously [7]. The primary breast cancer surgery had been conducted in 1974-2006. The cases were selected depending on the time lapse from the primary operation to the first recurrence to represent quick (</= 2 years), intermediate (5-10 years) and slow (>10 years) progressors, and three groups were formed: Group 1 n=41, tumors with R within two years after primary surgery, Group 2 n= 57, with R within 5 -10 years and Group 3 n=39 with R after 10 years or later (range >10 to 23 years). The archival slides were re-examined, and the histological tumour type and grade were assigned based on the criteria of Elston and Ellis [8]. The clinicopathologic characteristics of the patients and their cancers are summarized in Table 1.

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Table 1: Clinicopathologic parameters of the 137 breast cancer patients and the site of recurrence

	Group 1 n=41	Group 2 n=57	Group 3 n=39
Age at surgery of primary tumor			
< 50 years	19	20	18
≥ 50 years	22	37	21
Tumor size			
≥ 20 mm	14	28	24
< 20 mm	26	28	15
system missing	1	1	
Lymph node			
negative	14	34	21
positive system missing	24 3	20 3	13 5
Grade			
1	4	7	8
2	22	35	26
3	15	15	5
Histological type			
ductal	24	36	16
lobular	17	19	23
special types	0	2	0
Tissue site of Recurrence			
skin	6	10	11
soft tissue	6	12	5
subcutaneous tissue	12	16	15
lung	0	4	2
brain	2	2	0
lymph node	2	1	2
ovary	0	1	0
bone	3	6	4
liver	5	2	0
pleura	0	1	0
peritoneum	2	1	0
mesenterium	1	0	1
larynx	1	0	0
uterus	1	0	1
duodenum	0	1	0

Group1: dormancy of 0-2 years; Group 2: dormancy of 5-10 years; Group 3: dormancy of > 10 years

The Ethical Committee of the University Central Hospital of Helsinki approved the study protocol.

Immunohistochemistry

Four μm thick sections from paraffin blocs were deparaffinized in xylene and rehydrated. To block endogenous peroxidase, the slides were treated in a PT module (LabVision UK Ltd, Suffolk, UK) in Tris–HCl buffer (pH 8.5) for 20 min at 98 °C and with 0.3% Dako REAL Peroxidase Blocking Solution for 15 min. Immunostaining was performed in an Autostainer 480 (LabVision Thermo Scientific, UK Ltd, Cheshire, UK) by addition of the primary antibodies, see below, followed by 30 min incubation with Dako REAL EnVision/HRP detection system, Rabbit/Mouse (ENV) reagent (Dako, K5007), and the visualization of staining was done by Dako REAL DAB+Chromogen (Dako, K5007) for 10 min. Washing with PBS-0.04%-Tween20 took place between each step. Both stainings were counterstained with Mayer's hematoxylin and mounted in mounting medium. As primary antibodies the following reagents were used: 1.Rabbit polyclonal antibody against AZIN1, raised using a recombinant human AZIN1 peptide 250-448 aa, dilution 1:500 (Catalog Number: orb154904, Biorbyt Ltd. United Kingdom) 2. Rabbit polyclonal antibody to AZIN2, dilution 1:800, made against a synthetic peptide (STRDLLKELTLGASQATT) corresponding to amino acids 18–36 of AZIN2 (Genbank accession no. NP_443724). This N-terminal sequence covers those splice variants of AZIN2 which contain exons 1 and 2, and has a low homology to ODC and AZIN1 (14 and 5%, respectively).

The level of staining in cancer- and benign epithelial cells was scored as negative 0, faint 1/2-1+, moderate 2+ and strong 3+.

For monitoring ER we used anti-ERalfa clone 6F11 at dilution 1:50 (Novo Castra Newcastle, UK), and for PR anti-PR alfa clone 636, at dilution 1:100 (DacoCytomation, Denmark).

HER2 at protein level was monitored using anti-HER2 clone CB11 at dilution 1:400 (Novo Castra, UK). Ki67 was monitored using anti-Ki67 clone MIB-1 at dilution 1:75 (Daco Cytomation, Denmark) as primary antibody.

The expression of ER, PR, HER2, and MIB1 was recorded as percentage of positive tumor cells (range 0-100%) [7, 9]. All tumors with over expression of HER2 at protein level (2+ and 3+; see ref. 7) were tested for HER2 gene amplification by Inform HER2 Dual ISH test (inform HER2 dual in situ hybridization). The HER2 gene was targeted by a dinitrophenyl labeled probe (INFORM HER2 Dual ISH DNA Probe Cocktail, 780-4422, Roche/Ventana/).

Human skin with normal sweat glands was used as positive control for AZIN1 and AZIN2. For ER, PR, Ki67 and HER2 normal and malignant breast tissue was used. Specimen processed without primary antibody served as negative control.

We evaluated the entire tumor area from one representative section from the PT sand the Rs. The results were scored independently by three pathologists (KJ, MH, PH).

Statistical methods

All statistical analyses were performed using SPSS 24.0 for Windows (SPSS Incorporation, Chicago, IL, USA). The differences between the expression of the markers in Pts and the corresponding Rs within the groups were tested using paired samples t test. Differences between Groups and malignant versus benign epithelium were tested with independent t test. Kruskal-Wallis test and the Mann-Whitney U test was used to detect dissimilarity amongst the Groups. For analyzing the association of the expression of the markers with the clinicopathologic parameters and the established clinical breast cancer markers ER, PR, Ki67, and HER2, we used the categorical two-tailed Pearson's chi-square test. For Pearson's chi-square tests the cutoff point for negativity versus positivity

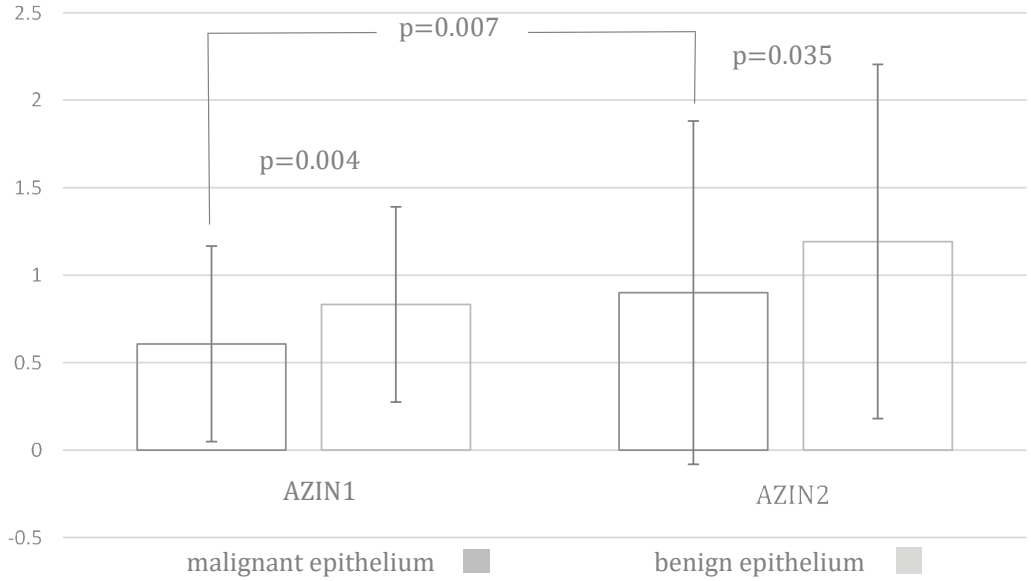

Figure 1: Expression of AZIN1 and AZIN2 at protein level in benign and malignant epithelial cells in Pts.

was < + 2 versus ≥ + 2 for AZIN1 and AZIN2. For ER and PR the cutoff point for positivity was 1%, and for Ki67 ≥ 14%.

For HER2 only those tumors with positive gene amplification were considered positive.

Probability values $p < 0.05$ were considered significant except in the Mann-Whitney U test, where $P < 0.0167$ (< 0.5/3) was considered significant.

Results

AZIN1 vs AZIN2

The overall expression of AZIN1 at protein level in malignant epithelial cells in Pts was lower than that of AZIN2 (p=0.007, Figure 1). Both AZIN1 and AZIN2 were expressed at a higher level in benign breast or other glandular epithelium in the samples of Pts than in the malignant breast epithelium (p=0,004 and 0,035 respectively, Figure 1, 2). In Rs benign epithelium was seldom present, and therefore no statistical analysis of the differences in expression was performed.

The intracellular localization of AZIN1 differed from that of AZIN2: the latter was concentrated on nuclear cell membrane and in small granules around the nucleus, whereas AZIN1 showed an even cytoplasmic staining with a faint rim on the outer cell membrane (Figure 2).

AZIN1

The expression of AZIN1 in the malignant epithelium tended to be the lower in the primary tumor samples the later the recurrence was recorded, although the difference was not statistically significant by Kruskal-Wallis test (Table 2 and 3, Figure 3). In benign epithelium the same tendency was seen, the fastest progressing tumors (Group 1) having a higher expression level as compared to the slower progressors (Group 2 and 3) (Figure 3).

When analyzing the Groups together, the expression of AZIN1

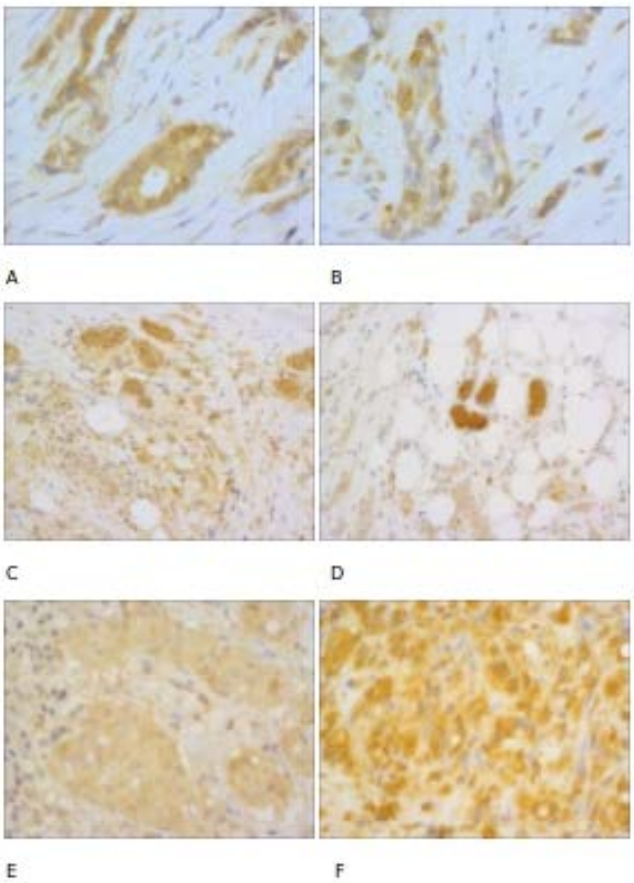

Figure 1: A – B: difference in the subcellular localization of AZIN1 (A) and AZIN2 (B) in malignant breast cancer epithelium.
C – D: strong staining in benign glandular cells as compared to malignant epithelial cells for AZIN1 (C) and AZIN2 (D).
E – F: staining for AZIN1 in Pt (E) is lower than in the corresponding R (F); a representative pair of samples from the Group 3.

Table 2: Comparison of the 3 Groups with regard to the mean expression of AZIN1 in malignant epithelium in PTs and corresponding Rs, Kruskal-Wallis test

	Group	n	Mean Rank	p
AZIN1 PTs	1	36	63,04	
	2	50	52,88	
	3	26	54,40	0,298
AZIN1 Rs	1	32	47,73	
	2	48	53,60	
	3	28	67,75	0,044

Kruskal-Wallis test showed a stochastical dominance by one Group over another with regard to expression of AZIN1 in Rs.

Table 3: Comparison of the 3 Groups with regard to the mean expression of AZIN1 in malignant epithelial cells in Rs, Mann-Whitney U test

AZIN1 expression (0-3) in malignant epithelial cells in Rs	Group 1 (n=32) Mean (SD)	Group 2 (n=49) Mean (SD)	U	Z	P
Group 1 versus Group 2	1,0938 (0,58802)	1,2041 (0,70651)	705,500	-,844	0,399
	Group 1 (n=32) Mean (SD)	Croup 3 (n=28) Mean(SD)			
Group 1 versus group 3	1,0938 (0,58802)	1,5000 (0,50918)	294,000	-2,620	0,009
	Group 2 (n=49) Mean (SD)	Croup 3 (n=28) Mean (SD)			
Group 2 versus group 3	1,2041 (0,70651)	1,5000 (0,50918)	539,500	-1,723	0,085

According to Mann-Whitney U test, the mean expression of AZIN1 was significantly higher in Rs in Group 3 than in Group 1. P value was considered significant when <0,0167 (<0,05/3).

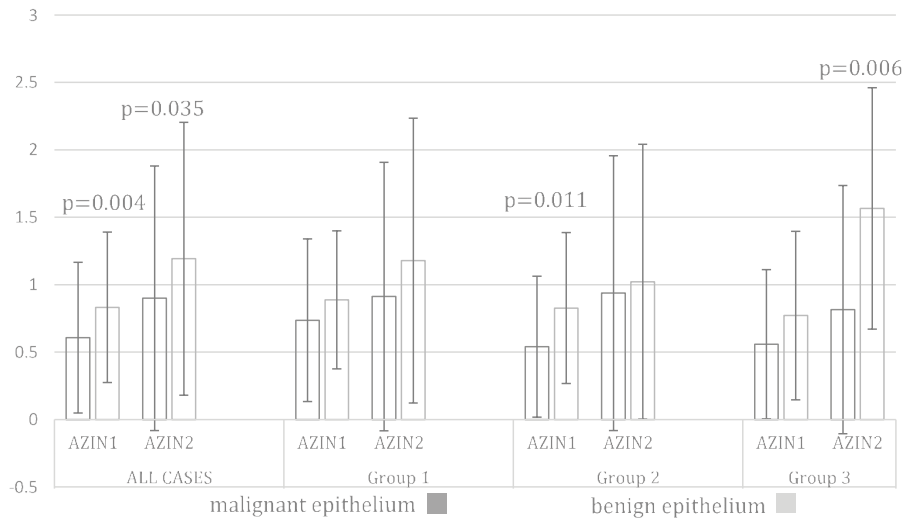

Figure 3: The means of the expression of AZIN1 and AZIN2 at protein level in benign and malignant epithelial cells (0, 1/2+,1+, 2+, 3+) in the Pts by Group.

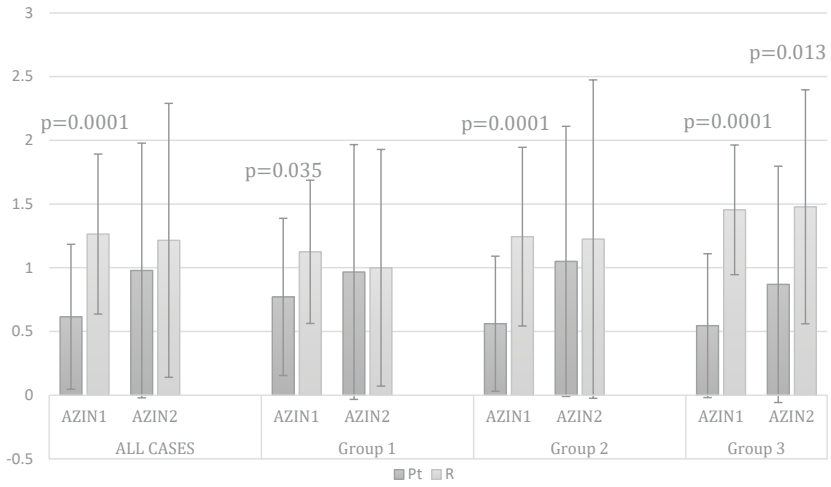

Figure 4: The means of the expression of AZIN1 and AZIN2 at protein level in malignant epithelial cells (0, ½+,1+, 2+, 3+) in the PTs and their corresponding Rs.

Table 4: Correlation of the expression of AZIN1 and AZIN2 with the protein level expression of ER, PR and Ki67, HER2 gene amplification and clinicopathological parameters in PTs of the 137 breast cancer patients. Significant results are shown.

	CishH neg N (%)	CishH pos N (%)	p
AZIN1 neg N (%)	87 (87,0)	13 (13,0)	
AZIN1 pos N (%)	3 (50)	3 (50)	0,014
	PR neg N (%)	PR pos N (%)	p
AZIN1 neg (%)	30 (29,4)	72 (70,6)	
	5 (71,4)	2 (28,6)	0,021
	PR neg N (%)	PR pos N (%)	
AZIN2 neg N (%)	29 (38,2)	47 (61,8)	
AZIN2 pos N (%)	5 (16,1%)	26 (83,9)	0,026

AZIN1 and AZIN2 neg = expression <2, pos = expression 2-3

in malignant epithelium was significantly higher in the recurrent tumors than in the primary tumors (p=0,0001, Figure 4, also illustrated in Figure 2). In Groups analyzed separately the difference was smaller in Group 1 (p=0,035, Figure 4), where the expression was relatively high in primary tumors. In Group 2 and 3 (p=0,0001 in both, Figure 4) the difference was larger primarily due to the lower expression in primary tumors. There was, however, a significant difference between Group 1 and Group 3 in the expression in Rs: the expression was higher in the late recurring cases (Table 2 and 3).

The expression of AZIN1 correlated positively with HER2 gene amplification and PR expression (p=0,014, p=0,021, respectively, Table 4).

AZIN2

AZIN2 was expressed approximately at the same level in malignant epithelium in Pts in all Groups, with a slight tendency of lower expression in the latest recurring tumors (Group 3); Kruskal-Wallis test found no dominance (data not shown). The difference in the expression between Pts and the corresponding Rs was not significant in all cases analyzed together (Figure 4), and was negligible in Groups 1 and 2. However, it reached significance in the late recurring tumors (Group 3, p=0,013, Figure 4).

A high expression of AZIN2 in primary tumors correlated positively with expression of PR (p=0,026, Table 4).

Discussion

Polyamines are crucial for proliferation, and in that role they function in diverse processes including regulating chromatin condensation, stabilizing the double helical structure of DNA, and regulating translation through a unique post-translational modification known as hypusination [10]. Elevated polyamine levels stimulate cellular proliferation and angiogenesis in tumors [2, 11-13], but they also exert an immunosuppressive effect by inhibiting pro inflammatory cytokine synthesis and directing myelous suppressor cells and macrophages to obtain a tumor supporting phenotype [14 -20].

The regulation of the availability of polyamines in the cell is strongly regulated. Ornithine decarboxylase (ODC), the rate-limiting enzyme responsible for their synthesis, and the ODC-inhibiting antizymes (AZIs) and AZI inhibitors (AZINs), form a complex network of factors aiming at optimizing the polyamine balance for the ever changing cellular needs. A high level of polyamines supports proliferation, and it is a common feature in malignancies. The activity of ODC is crucial for polyamine synthesis, and AZINs with their capability of negating the action of the ODC-inhibiting AZs are important players in determining their synthesis-based availability. Indeed, AZIN1 is emerging as an oncogene and a potential target in clinical cancer care due to its ODC activity boosting capacity. The human AZIN1 gene has been located at chromosome 8q22.3, a frequently amplified hot spot associated with poor prognosis and accelerated distant metastases in breast tumor (21). A to I RNA editing of AZIN1 is intimately associated with augmented tumor-initiating potential [21-23]. AZIN1 has also been reported to have direct effects on the regulation of cell cycle and apoptosis [24-28].

In our study the expression of AZIN1 at protein level in Pts was the higher the sooner the tumor relapsed, which is consistent with the previous data associating high expression of AZIN1 with a high potential of proliferation and metastatic spread [21]. Also benign epithelium in the samples of Pts had a higher expression level in quickly relapsing tumors than in those with a long dormancy. Notably, the expression was higher in normal epithelium than in malignant. The induction of AZIN1 RNA in response to growth stimuli has been shown to happen earlier than for instance that of ODC [29], and the high expression in benign cells might reflect an early stimulus for creating and maintaining a local pro-tumor environment.

The expression of AZIN1 was significantly higher in Rs than in the corresponding Pts, reflecting a shift in the grade of malignancy, possibly due to new mutations in the surviving tumor cells. The difference between Pts and Rs was larger in the late recurring tumors, mainly because of the relatively low expression in the slowly progressing Pts. The expression was significantly higher, though, in Rs in Group 3 than in Group 1, suggesting that a higher expression of AZIN1 might be required for tumor progression after a long dormancy than after a short one.

A high expression of AZIN1 in Pts correlated positively with HER2 gene amplification and PR expression.

An over expression of HER2 has originally been recognized as an inherent marker of early progression and inferior overall survival in early breast cancer. Although targeted treatment has changed the outcome [30], the involvement of HER2 gene amplification in tumor initiation refers to an aggressive disease with a high cell division rate, and a concomitant high expression of AZIN1 can be considered related to this.

Survival is shorter in patients with ER-/PR+ breast cancer than in those with ER+/PR+ cancer [31]. However, expression of PR is rare in the absence of ER, indicating that the expression of AZIN1 largely associated with an ER+PR+ phenotype. Hormone receptor positive tumors have in general a better prognosis than hormone independent (31). The specific role

of PR is poorly characterized, but it has been suggested that progestins may modify the ER pathway via PR, and this modification may be beneficial for the prognosis [32]. An association of ER+PR+ phenotype with a high expression of AZIN1, a poor prognostic sign, hence seems unexpected, maybe reflecting the fragmentary understanding of the functional role of PR in breast cancer [32].

AZIN2, a more recently recognized member of the AZ inhibiting protein family, has multiple structural and functional similarities to AZIN1, but its role in the maintenance of the balance in normal and malignant cells is not entirely understood. AZIN1 is ubiquitously expressed at high levels, whereas the expression of AZIN2 is more restricted. AZIN2 was first identified as an ODC paralogue [33], and was later shown to be a tissue-specific antizyme inhibitor in the brain and testes [34, 35]. Later AZIN2 was also shown to be expressed in terminally differentiated secreting cells in various other organs [6, 36, 37]. AZIN2 binds to the membranes of the trans-Golgi network (TGN), which is a dynamic composition of a tubular membrane network regulating secretion and intracellular macro molecule traffic. Polyamines seem to be involved in membrane trafficking [38, 39], and AZIN2 might act as a local activator of ODC, regulating secretion and excretion [36, 39, 40]. A fraction of ODC translocates to the cell membrane during cell activation and transformation via the p47 phox-related membrane targeting motif [41], conserved in AZIN2 but not in AZIN1 [33, 37]. In our study AZIN2 was expressed at protein level in nuclear membranes and intracellular granules, consistently with the previous data. It was expressed at a higher level in benign epithelium than in malignant, probably reflecting a difference in macro molecule transport in normal versus dysorganized malignant cells. There was negligible difference in the expression in Pts between the Groups, suggesting that AZIN 2 does not contribute to nor reflect the aggressiveness of the tumor. There was, however, a tendency of a higher expression in Rs versus Pts. This might have been induced by a growing intracellular trafficking or a difference in the tumor micro environment [42], largely orchestrated by the immune system. The increase of expression was significant in Group 3, and hence a connection with tumor progression is not fully excluded although not stronly supported by these data. A high expression of AZIN2 in Pts correlated with the expression of PR, the significance of which remains elusive due to the incomplete understanding of the role of PR in breast cancer development.

The biological behavior of tumors does not depend on the features of the malignant cells only: the tumor micro environment including the immune response towards the invading abnormality is an important part of the game. Accordingly, the action of AZINs in tumors must be seen as the result of their effect on both of these entities. In tumor cells a high expression of AZIN1 and consequent high amount of polyamines predicts fast proliferation. In the tumor environment polyamine rich environment promotes pro-tumor M2 macrophages, characterized by the expression of arginase, which hydrolyzes arginine to ornithine and urea, over anti-tumor M1 macrophages expressing nitric oxide synthase, which metabolizes arginine to nitric oxide (NO) and citrulline [43, 44], and attenuates the anti-tumor functions of T cells (45). We studied the connection of AZIN1 and AZIN2 expression in benign and malignant epithelial cells in Pts with disease outcome, measured by the length of tumor dormancy after the primary operation. In this simplified approach the mechanisms behind the connections could not be revealed, but previous findings by us and others suggest that the response of the tumor micro environment to the levels of AZINs and hence levels of polyamines has an important role in regulating tumor progression [19, 42-44].

Conclusion

Our results support the consensus of a high availability of polyamines supporting tumor progression and metastatic spread. The data suggest that a high level of AZIN1 in the primary tumor, most likely by increasing the availability of polyamines in the tumor cells and tumor stroma, predicts an early progression of breast cancer. The expression of AZIN1 is higher in metastatic lesions than in primary tumors, suggesting that it may be one of the initiating incentives for tumor spread. On the contrary, AZIN2 did not seem to have a clear correlation with the behaviour of the tumor.

AZIN1 is a potential prognostic marker and target for intervention in both early and disseminated breast cancer. AZIN1 and the downstream players in the immune system are more feasible targets for intervention than epitopes from tumor cells, which are under a constant pressure to mutations.

Acknowledgments

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