Does thymidine phosphorylase correlate with angiogenesis in intraductal carcinoma of the breast?

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ABSTRACT

Objectives: To determine the association between thymidine phosphorylase (TP) and angiogenesis, and other conventional prognostic markers. We also evaluated interobserver and intraobserver reliability for TP expression in ductal carcinoma, to achieve a more consistent results.

Methods: Our study included all cases diagnosed in Adnan Menderes University Medical Faculty Hospital, Aydin, Turkey as invasive ductal carcinoma or ductal carcinoma in situ (DCIS) with proven component of (>30%), between January 2003 and February 2005. The total number of the cases was 27 and their median age was 50 years. All sections were stained using monoclonal antibody-TP and examined at ×40 magnification. Either nuclear or cytoplasmic staining was accepted as positive. The histoscore (H-score) was calculated for each specimen. The tumor stromal vascularity was assessed by monoclonal anti-CD34; and areas of intense vascularization were determined. Conventional immunohistochemical markers such as c-erb B2, Ki-67, estrogen and progesterone receptors and p53 were also applied to all slides. Three pathologists blindly examined each slide under 10 high-power fields (10 HPF) for 2 times in a 2 months period.

Results: There was no significant association between stromal vascularity and TP staining of cancer cells (p=0.1) and no correlation was determined between H-scores for TP staining in ductal carcinoma and DCIS components (p=0.5).

Conclusion: There was no significant correlation noted between stromal and periductal vascularity with the anti-CD34 antibody was used. No significant correlation was identified between the TP H-score and stromal or periductal vascularity.

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Thymidine phosphorylase (TP) is an enzyme that reversely catalyses the dephosphorylation of deoxyuridine, thymidine and their analogs. Thymidine phosphorylase, also known as platelet-derived endothelial-cell growth factor (PD-ECGF), activates the oral cytostatic drug 5 -deoxy-5 -fluorouridine and the intermediate metabolite capecitabine.[1] The TP has angiogenic activity in human solid tumors and has to play a role in endothelial cell differentiation and migration.[2]

Thymidine phosphorylase plays a role in endothelial cell differentiation and migration and has an angiogenic activity in human solid tumors. The exact mechanism on how TP promotes angiogenesis is

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still unknown. This mystery has gained much interest both in clinical and pathological practice. There is accumulating evidence supporting the theory that angiogenesis has a prognostic role in breast cancer. Angiogenesis is considered to be strongly associated with early local recurrence and distant metastasis.[3] The TP is chemotactic and non-mitogenic for endothelial cells and induces angiogenesis in vivo and in wound healing.[4] The TP activity can be easily determined by biochemical studies. Biochemical methods such as liquid chromatography or enzyme-linked immunosorbent assay has confirmed that TP activity in tumor tissue is higher than normal tissue.[5] It also suggests that the amount of TP activity of cancer cells is different from stromal cells. Immunohistochemical studies revealed that the clarification of the cell specific role of TP would be both appropriate and beneficial. The reliability between observers was analyzed to overcome subjective nature of immunohistochemical methods. This has not only secured an accurate evaluation, but also helped to determine whether TP immunostaining was reproducible.

The aim of the present study was to determine whether TP staining is associated with angiogenesis or other conventional immunohistochemical prognostic markers.

Methods. All cases of invasive ductal carcinoma or ductal carcinoma in situ (DCIS) with a proven (>30%) component, diagnosed in Adnan Menderes University Medical Faculty Hospital, Aydin, Turkey between January 2003 and February 2005 was included in our study creating 32 cases initially. Mastectomy and ipsilateral axillary lymph node dissection were performed to all cases. Five cases lost throughout the follow-up period were excluded from the study leaving 27 cases with a median age of 50 years.

Immunohistochemistry. Sections with 4 μm-thickness obtained from formalin-fixed, paraffin-embedded tissues were placed on coated slides. Immunostaining was performed using the avidinbiotin complex method. After deparaffinization and dehydration, sections in citrate buffer (0.01 mol/L, pH 6.0) were treated in a microwave oven at 700 W for 2 times each lasting for 5 minutes. Then slides were left for one hour to allow cooling to room temperature. Sections were immersed in 3% hydrogen peroxide in methanol for 30 minutes to inhibit endogenous peroxidase activity and then incubated with primary antibody at room temperature for one hour. After this incubation, biotinylated goat anti-rabbit secondary antibody was added and the solution was kept at room temperature for another 60 minutes. Bound antibody was observed by avidin–biotin–peroxidase complex (Histostainplus Kits, Zymed, San Francisco, USA, code no: 85- 9843) for one hour at room temperature. Color developed by 3,3'-diaminobenzidine tetrahydrochloride. Between these steps, slides were rinsed 3 times in tris-buffered saline (pH 7.6) for 10 minutes. The slides were lightly counterstained in Harris' hematoxylin, and then dehydrated and mounted. The antibodies used were as follows: estrogen receptor (ER), (Novocastra, Newcastle, United Kingdom, code no: NCL-L-ER-6F11), 1/80 dilution, progesterone receptor (PR), (Novocastra, Newcastle, United Kingdom, code no: RTU-PGR-312), c-erbB2, (Neomarkers, CA, USA, code no: MS-730-R7), p53,(Novocastra, Newcastle, United Kingdom, code no: RTU-p53-DO7), Ki-67, (Novocastra, Newcastle, United Kingdom, code no: RTU-Ki67-MM1), CD34 (Neomarkers, CA, USA, code no:MS-363-R7) and TP (Neomarkers, CA, USA, code no:MS-499-R7). Through the immunohistochemical staining period, internal positive control, as well as various tissue samples, was used. For negative control, primary antibody phase was skipped to continue with the staining process.

Evaluation of TP staining. Three pathologists independently examined all sections under ×40 magnification. Either nuclear or cytoplasmic staining was accepted as positive. Both nuclear and cytoplasmic TP expression was observed in the cases but especially cytoplasmic staining was predominating. Positive stained cells in all preparations exposed to immunohistochemical staining were evaluated by Histoscore (H-score), which identifies ER positiveness and thus, regarded as a more reliable method. Nuclear staining for ER, PR, p53, Ki-67, cytoplasmic membrane staining for C-erbB2, and nuclear or cytoplasmic staining or both for TP of at least 300 cells, where 100 of them belong to 3 different areas namely, most intensive staining, medium staining, most weak staining, were counted to calculate the percentages of stained cells. The percentages of cells were multiplied with 3 in the area showing most intensive staining; was multiplied with 2 in the area showing medium staining and multiplied with one in the area showing the weakest staining and the sum of these multiplications were calculated.

Evaluation of stromal vascularity of the tumor. Areas with high vascularization were solely selected throughout the stromal areas that were nearest to the edge of the tumor, whether DCIS or invasive ductal carcinoma. Samples examined under 10 high power fields (×200) magnification and presence of red blood cells was used as the only criterion to determine the vascularization. Standard deviation was calculated.

Evaluation of interobserver and intraobserver reliability. Slides were independently examined for 2 occasions under 10 high power fields, and a total of 100 cells were counted. Score of each slide was expressed as percentages (%). Intraobserver reliability levels were assessed by randomly selected slides. Our resident who did not join in the scoring process carried out the slide selection process. Two months after, observers reassessed the slides again in a blind fashion. The difference among the observers was analyzed by Friedman test.

There was no significant correlation between stromal and periductal vascularity when anti-CD34 antibody was used. No significant correlation was identified either between the TP H-score and the stromal or periductal vascularity.

Statistics. Patients and tumor staining data were analyzed by minimum, maximum and median values. The correlations between the TP levels and ER, PR, p53, Ki67 and tumor grade values were analyzed by Spearman's Correlation. A "*p*" value less than 0.05 was considered significant. The Statistical Package for Social Sciences software (version 10.0, SPSS, Chicago, IL) was used for the statistical analysis. The interobserver and intraobserver differences on the TP staining levels were assessed by Friedman test.

Results. Patient characteristics and staining ratio of the TP and CD34 of the specimens with minimum, maximum and median values are shown in **Table 1**. Immunohistochemical staining characteristics of the tumors are given in **Table 2, Figures 1 & 2**. No correlation was found between the TP and CD34 staining levels (r: 0.21, *p*=0.28). Similarly, TP levels were not correlated with age (r: 0.13, *p*=0.51), tumor diameter (r: 0.19, *p*=0.53), lymph node metastasis, (r: -0.02, *p*=0.89). The TP staining characteristics were also not correlated with the ER (r:0.18, *p*=0.36), PR (r:-0.38, *p*=0.05), p53 (r: 0.13, *p*=0.50), Ki67 (r:-0.05, *p*=0.79), cerb B2, (r:0.13, *p*=0.50) and tumor grades (r: 0.04, *p*=0.85) staining characteristics. Significant correlation was found in between ER and PR status (r: 0.50, *p*=0.008) and in between p53 and tumor grade (r:-0.63, *p*=0.001). Interobserver counting values of the TP and CD34 staining characteristics are given in **Table 3**. No interobserver differences was found for TP staining (*p*=0.50). Also, intraobserver differences were not found for TP staining (*p*=0.865). Intraobserver count results were given in **Table 3**.

Discussion. Thymidine phosphorylase is involved in the degradation of pyrimidine nucleosides through phosphorolysis. The expression level of TP is usually higher in human solid tumors such as the

Table 1 - Median values of the patients' age, tumor diameter, node metastasis and positive stained cells with the anti-thymidine phosphorylase and anti-CD34.

Parameters	Median	Minimum	Maximum
Age (year)	50	37	66
Tumor diameter (cm)	3	1	11
Lymph node metastasis (n)	0	0	43
Thymidine	10	1	90
CD34	6.5	4	10

Table 2 - Staining characteristics of the study group.

Characteristics	N	(%)
Invasive / ductal carcinoma in situ	23 / 4	15 / 85
Grade		
Low	8	(35)
Intermediate	12	(52)
High	3	(13)
Estrogen receptor status		
<5%	6	(22)
5-20%	8	(30)
20-50%	9	(33)
>50%	4	(15)
Progesterone receptor status		
<5%	5	(18)
5-20%	14	(52)
20-50%	7	(26)
>50%	1	(4)
Cerb-B2		
<5%	4	(14)
5-20%	8	(30)
20-50%	8	(30)
>50%	7	(26)
p53 status		
<5%	11	(41)
5-20%	9	(33)
20-50%	5	(19)
>50%	2	(7)
Ki-67 status		
<5%	6	(22)
5-20%	13	(48)
20-50%	5	(19)
>50%	3	(11)

Table 3 - Interobserver and intraobserver counting values of the thymidine phosphorylase (TP), and interobserver counting values of CD34 staining characteristics.

Observers	First counting values of TP (Min - Max)	Second counting values of TP (Min - Max)	Counting values of TP (Min - Max)
1. Observer	10 (1 - 90)	10 (1 - 90)	6.5 (4 - 10)
2. Observer	10 (0 - 95)	15 (0 - 90)	6.7 (5 - 10)
3. Observer	20 (0 - 90)	22.5 (0 - 90)	6.8 (5 - 11)

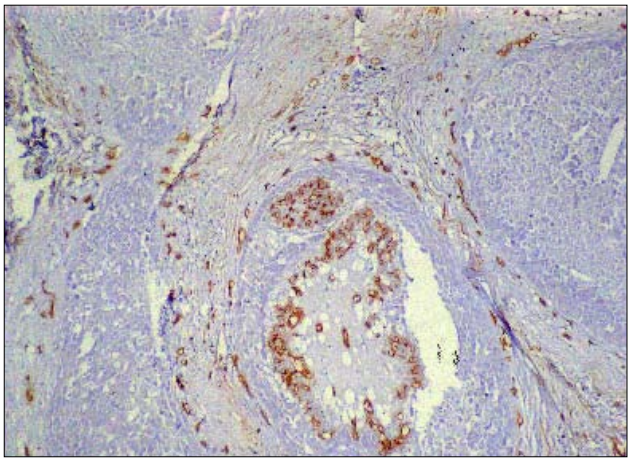

Figure 1- Periductal stromal vascularity (anti-CD34 immunostaining × 100).

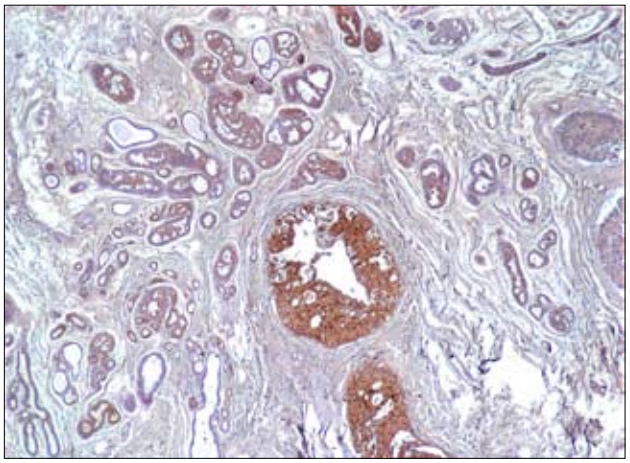

Figure 2- Intraductal epithelial expression of anti-thymidine phosphorylase × 100.

breast carcinomas. Not only TP, but also thymidylate synthase and dihydropyrimidine dehydrogenase have been suggested as predictive markers for epithelial malignancies.[6] The TP expression is expected to increase in DCIS and invasive ductal carcinomas but clinical studies yield conflicting results. Some authors have suggested that TP has angiogenic effects in breast carcinomas, but TP was found out to have a minimal role on tumor angiogenesis in some studies.[7,8] Angiogenesis is believed to have an important role in cancer progression.[9] Angiogenesis also has a paramount importance in the development of hyperplastic and precancerous lesions.[10] Two vascular patterns have been described in ductal carcinoma and in situ lesions: increased stromal vascularity and increased periductal blood vessels.[2] Teo et al, stated that an increase in the periductal mean vascular density around DCIS can predict the development of a recurrence, particularly if it has an invasive nature.[11] Other factors related to angiogenesis, such as vascular endothelial growth factor (VEGF) have more positive effects.[12] Teo et al[11] also noted that vascular endothelial growth factor receptor-3 was more prominent in the vessels adjacent to the basal lamina of the affected ducts.

Qualitative and semiquantitative assessments of periductal vascularity were studied by several authors.[8,13,14] They defined periductal vascularity as the necklace of vessels that are nearest or within a defined distance from the basement membrane. Importance of these 2 patterns is still unclear.[7,13,14] We have used qualitative method, so both periductal and stromal vessels were evaluated.

Guidi et al[13] showed a significant association between high Ki-67 proliferation indexes and high microvessel counts.[13] Teo et al[11] stated that an increase in microvessel density around DCIS could not predict the development of recurrence of either invasive or in situ lesions.[11] They also suggested that TP expression does not seem to be related with recurrence after operation. Toi et al[15,16] demonstrated that invasive breast carcinomas has a significant correlation between microvessel density and both TP and VEGF. In this study, there was no significant correlation noted whether between stromal and periductal vascularity with the anti-CD34 antibody nor was identified between the TP H-score and stromal or periductal vascularity. A number of different studies support that exogenous and endogenous steroids, particularly estrogen, have a negative role in the development of breast cancer. It is known that steroid receptors, which play a role in the development of ductal epithelium, are also effective in the development of cancer through various ways. Effect on apoptosis via bcl-2, such as c-erbB2 on proto-oncogenes, which regulates cell cycle, and directly on cell DNA, which has been determined recently, can be considered among these effects.

Tsuda et al[17] suggested that 3 or more observers should participate in the evaluation of TP expression. They also proved that TP immunostaining was reproducible and for a consistent calibration and more reliable and accurate results both the majority system and the total scoring system appeared to reflect all observers' scores and to minimize bias.[17]

In conclusion, no correlation was found between the TP and CD34 staining levels. Similarly, TP levels were not correlated with age, tumor diameter, and

lymph node status. The TP staining was also not correlated with the ER, PR, P53, Ki67, c-erbB2, and tumor grade. But, a significant correlation was found in between ER and PR status and in between p53 and tumor grade. No interobserver and intraobserver differences were found in the TP staining. There was no significant correlation noted between stromal and periductal vascularity with the anti-CD34 antibody used and also between the TP H-score and stromal or periductal vascularity. It was strongly suggested that 3 or more rates should evaluate the staining results in order to acquire stable and reliable results in TP expression evaluation. We have found out that TP expression by immunohistochemistry was reproducible. The determination of the true impact of TP expression will require studies with larger patient groups and more patient events.

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