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Are MIB-1, Her2/neu or CD34 useful prognostic factors in stage II and III testicular non-seminomatous germ cell tumors?

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SUMMARY

In this study we analyzed immunocytochemical expression of MIB-1, HER2/neu and CD34 in stage II and III testicular non-seminomatous germ cell tumors to investigate if these factors may be useful in determining the prognosis of the disease. Orchiectomy specimens of 64 patients were studied for MIB-1, HER2/neu and CD34 immunostaining. In 47% and 30% of the cases MIB-1 immunostaining level was under the median value in patients with stage II and III disease, respectively. The intensity of immunostaining with CD34 was (+) in 31% of the cases, (++) in 40% of the cases and (+++) in 29% of the cases in stage II tumors. The intensity of CD34 immunostaining was (++) in 26% and (+++) in 73% of the patients with stage III tumors. None of the specimens were stained with HER2/neu. The intensity of CD34 and MIB-1 immunostaining increased with advancing stage. This study revealed that MIB-1, CD34 or HER2/neu immunostaining are not good prognostic factors to predict the survival in patients with stage II and III testicular non-seminomatous germ cell tumors.

Key words: *CD34, HER2/neu, MIB-1, non-seminomatous, testicular germ cell tumor*

ÖZET

MIB-1, Her2/neu veya CD34 testisin evre II ve III non-seminomatöz germ hücreli tümörlerinde kullanışlı prognostik faktörler midir?

Bu çalışmada MIB-1, HER2/neu ve CD34 immünhistokimyasal ekspresyonunun testisin evre II ve III non-seminomatöz germ hücreli tümörlerinde ekspresyonuna bakarak, bu faktörlerin hastalığın prognozunu belirlemede yardımcı olup olamayacağını araştırdık. Altmış dört hastaya ait orşiektomi örnekleri MIB-1, HER2/neu ve CD34 immün boyanma ile incelendi. Olguların sırasıyla %47 ve %30'unda immün boyanma düzeyi evre II ve III olgularda medyan değerin altında bulundu. CD34 ile immün boyanma yoğunluğu evre II olguların %31'inde (+), %40'ında (++) ve %29'unda (+++) olarak bulundu. CD34 immün boyanma yoğunluğu evre III olguların %26'sında (++) ve %73'ünde (+++) olarak bulundu. Örneklerin hiçbirisi HER2/neu ile boyanma göstermedi. CD34 ve MIB-1 immün boyanma yoğunluğu ilerleyen evreyle birlikte artış göstermiştir. Bu çalışma MIB-1, CD34 veya HER2/neu immün boyanmasının evre II ve III testis non-seminomatöz germ hücreli tümör olgularında sağkalımı göstermede iyi birer prognostik faktör olmadığını göstermiştir.

Anahtar kelimeler: *CD34, HER2/neu, MIB-1, non-seminomatöz, testis germ hücreli tümör*

Introduction

Currently, testicular cancer is considered as a curable cancer and durable remission may be achieved with conventional chemotherapies, even in advanced stages. The survival rate is reported between 55% and 75% in those patients (1). Although conventional or salvage chemotherapies are applied, some patients do not achieve a remission and die due to the progression of their disease (2).

Up to date many prognostic factors have been investigated to define a correlation between the prognosis and survival. Among those factors, MIB-1 which is a nuclear antigen expressed in all phases of the cell cycle except G0 (3), is a monoclonal antibody and has been used to assess the proliferative activity in human tumors (4). It has been found in conformance with the prognosis in some human solid tumors, such as breast cancer (5), colorectal cancer (6) or prostate cancer (7). It has also been shown that MIB-1 is a significant marker to detect the low risk group of patients prone to development of metastasis (8). On the other hand HER2/neu, which is a membrane glycoprotein and belongs to the epidermal growth factor receptor (EGFR) family has been shown to have a crucial role in angiogenesis (9). The CD34 antigen is a transmembrane glycoprotein and is expressed on lymphohematopoietic progenitor cells, vascular endothelial cells, fibroblastic spindle cells and some cells in the neural tissue (10). CD34 reflects the vascular density of tumor and has been shown as a blood vessel marker (11). Although many studies have been performed concerning the expression of the CD34 antigen in hematopoietic, soft tissue and skin tumors, there are limited numbers of reports in regard to its expression in neoplasms of other organ systems (12,13). Two studies have reported that yolk sac tumor is the only germ cell tumor component positive for the expression of CD34 antigen (14,15). Therefore, the results of current data are somewhat

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still conflicting and no enough data are present yet, to show if MIB-1, HER2/neu or CD34 are good prognostic factors in testicular germ-cell tumors (8,15,16).

In the present study, we investigated the immunostaining pattern of MIB-1, HER2/neu and CD34 in clinical stage II and III testicular non-seminomatous germ-cell tumors and determined whether these factors may predict the outcome of the disease in advanced stage tumor.

Material and Methods

Patient characteristics: Patients' characteristics are detailed in Table I. A total of 64 patients (19-40 years old) with initial stage II and III testicular germ cell tumors were retrospectively analyzed. Patients with seminomas, extragonadal germ cell tumors or other malignancies were excluded from the study. Clinical staging was done according to AJCC Cancer Staging (17) with computed tomography of the chest, abdomen and pelvis at initial presentation of the patients. Serum AFP and β-HCG levels, initial localization sites of tumor, metastasis sites (lung versus other than lung) were also noted for each patient. By this way, it was noted that 38 patients had stage II disease and 26 patients had stage III disease.

Histopathological evaluation: All archival tissue blocks from each tumor were initially checked by hematoxylin and eosin-stained sections to select the representative block with available tissue for immunohistochemical staining. A 4-μm thick section from each formalin-fixed paraffin-embedded tumor was stained using the primary antibody against CD34 (1:100; Neomarkers, Fremont, CA, USA), c-*erb*B-2 / HER2/*neu* (1:200; Neomarkers, Fremont, CA, USA) and Ki-67 (1:100; Clone SP6, Neomarkers, Fremont, CA, USA). Immunohistochemical staining was performed by the labeled streptavidin-biotin method using UltraVision Large Volume Detection System (Cat # TP-060-HL, LabVision, Fremont, CA, USA) kit using autostainer (Labvision, Fremont, CA, USA) according to procedure briefly described below.

Table I. Patient characteristics

	Number	*Percentage (%)*	*Total number*
Age			64
< 20	5	8	
20-30	53	83	
>30	6	9	
Stage			
II	38	60	
III	26	40	
Occurrence of relapse			23
Stage II	9	15	
Stage III	14	24	
Location of relapse			
Lung	9	15	
Other than lung	14	24	
Number of death			
Stage II	5	9	
Stage III	12	21	

Immunohistochemical staining procedure was performed as follows: sections were deparaffinized and rehydrated in graded ethanols. After rinsing in distilled water, sections were microwaved for 5 minutes at 750 watts in 0.01 mol/L sodium citrate buffer (pH 6.0); this step was repeated three times. The slides were immersed in 3% H_2O_2 in distilled water for 5 minutes and then in blocking solution for 30 minutes to block endogenous peroxidase activity and unspecific binding sites, respectively. Sections were then rinsed in phosphate-buffered saline (PBS) and incubated at room temperature with the primary antibody for 60 minutes, followed by a rinse in PBS. Omitting the primary antibody performed negative controls. The sections were thereafter treated with biotinylated secondary anti-rabbit antibodies in a dilution of 1:200, and antibody-binding sites were finally visualized by avidin-biotin peroxidase complex solution, using AEC as a chromogen.

Immunohistochemically stained slides were reviewed by two pathologists. The percentage of stained cells and staining intensity were evaluated for *HER2/neu* expression. MIB-1 labeling index was measured morphometrically using a computer system composed of a personal computer, a light microscope with motorized stage (Zeiss Axioscope, Zeiss, Göttingen, Germany), a frame grabber card (Matrox Meteor), a digital camera attached to the microscope (Sony AVT Horn 3 CCD). Using KS 400 software, a semiautomatic, interactive "macro" (a batch file containing all of the instructions to accomplish a predetermined set of measurements) was prepared. During measurement, a section of tumor immunostained with MIB-1 was examined under ×2.5 magnification. At this stage, a suitable quadrangular area was selected interactively with the aid of the tools of the software. Then, the entire area selected under ×2.5 magnification was examined with an ×20 objective as consecutive non-overlapping fields. Measurement cycles were completed in the following order: acquiring the image, adjusting focus, superimposing randomly oriented grids and dots on top of the image, counting dots over stained nuclei and non-staiend tumor nuclei, respectively, and acquiring image of the next field. After completing the last of the preselected fields, the

dot counts summed up by the software and the results were shown in a message window as percent of MIB-1 labeled nuclei in the tumor. These results were recorded for each sample.

CD-34 immunostained slides were assesed for microvessel density. Briefly, the tumor section was scanned at low power, and subjectively the three areas with the highest number of discrete microvessel profiles were selected (hot-spots). One microscopic field (Leica DMLB; Leica, Wetzler, Germany) was identified within each hot-spot at x200 magnification providing a 0.754 mm^2 field size. All individual microvessel profiles within the applied circular microscopic field were counted. MVD was defined as the number of manually counted vessel profiles per mm^2 (vp/mm^2) taken as the average from the three hot-spot counts. Then, they are categorized into three group: (+), <50; (++), 50-70; (+++), ≥70.

Statistics: All values in the text were given as median (min-max) and percents. The differences between the groups were tested using Kruskall Wallis and Mann-Whitney U tests. The relationships between the variables were detected by Spearman's correlation test. The survivals of patients were estimated by Kaplan Meier method. Log rank test was used to compare the survivals of groups. Cox proportional hazard analysis was used to determine the prognostic factors in univariate and multivariate analysis. All statistical calculations were performed with SPSS 10.0 for Windows statistical software package (Chicago, IL, USA). P value < 0.05 was considered statistically significant in all analysis.

Results

Patients and HER2/neu, CD34 and MIB-1 immunostaining findings: Sixty percent of patients had stage II disease and 40% of patients had stage III disease. All pathologic specimens were stained for MIB-1 by immunohistochemistry. Median level of expression of MIB-1 was 32 (range: 0-55). MIB-1 level was under 32 in 18 patients (47%) with stage II disease and 8 patients (30%) with stage III disease; the level was ≥ 32 in 20 patients (53%) with stage II disease and in 18 patients (70%) with stage III disease (Table II). The median level of expression of MIB-1 was 30 in stage II and 34 in stage III tumors. The difference was found significant statistically (p: 0.018). CD34 immunostaining was positive in all specimens. The level of CD34 immunostaining was (+) in 12 patients (31%), (++) in 15 patients (4%), and (+++) in 11 patients (29%) in stage II tumors. This level was (++) in 7 patients (26%) and (+++) in 19 patients (73%) (Table II). None of these specimens were stained with HER2/neu.

Uni and multivariate cox proportional hazard modeling results: The expression level of MIB-1 was not found as a significant prognostic factor statistically at a median level of 32 (OR 1.20, 95% CI (0.47-3.04), p=0.70) (Table III). Similarly, CD34 expression was not shown as an important factor for prognosis statistically (OR 31.8, 95%CI (0.32-3127.8), p=0.14) in univariate analysis. Multivariate analysis was not performed due to the lack of significance in univariate analysis.

Discussion

High proliferation indices are reported as a risk factor for relapse in testicular germ-cell tumors (18). DNA flow-cytometry (19), DNA image cytometry (20) and proliferating cell nuclear antigen (PCNA) were used to determine tumor proliferation rate (21). However, the analysis of MIB-1 or HER2/neu by immunohistochemistry is considered as a more useful and less expensive method in some studies to give information about the cell proliferation activity in testicular germ-cell tumors (8,22-25).

Albers et al. investigated 76 patients with stage I germ cell tumors and emphasized that MIB-1 is a good prognostic parameter and that it could be used even to classify patients with low metastatic risk due

Table II. Distribution of the patients according to the evaluated prognostic factors

	Stage II (n)	*Stage II (%)*	*Stage III (n)*	*Stage III (%)*	*Total (n)*	*Total (%)*
MIB-1						
<32§	18	47	8	30	26	40
≥32§	20	53	18	70	38	60
HER2/neu (-)	38	100	26	100	64	100
CD34						
(+)	12	31	0	0	12	19
(++)	15	40	7	26	22	34
(+++)	11	29	19	73	30	47

§: Median value

Table III. Univariate cox hazard proportional analysis result

Prognostic factor	*Hazard ratio*	*95% Confidence interval*	*p value*
MIB-1	1.20	0.47-3.04	0.70
CD34	31.8	0.32-3127.8	0.14
HER2/neu	NA	NA	NA

to high expression level in metastatic cases (26). On the other hand, Heidenreich et al. and Data et al. failed to show that MIB-1 expression did correlate with pathologic stage in NSGCTs (16,23,27). In our study, we detected MIB-1 expression in all specimens. The median level of MIB-1 expression was found significant statistically according to stages. However, at a median level of expression, we found that MIB-1 was not a significant prognostic factor in univariate analysis. In various studies, investigators have selected different cut-off value for MIB-1, which made difficult the interpretation on risk factor analysis (13). The cut-off value of Albers et al. was 70% (8). We selected the mean value 32% as a cut-off value. The difference in results between the Albers' and our study may be related to the cut-off value selected (28).

CD34 has been shown as a blood vessel marker in various tumors and is used to identify the vascular density of the tumor (11). It has been shown that quantitative evaluation of angiogenesis by CD34 immunohistochemistry may be helpful to differentiate hepatocellular carcinoma (HCC) and non-HCC. However, the authors emphasized that no prognostic information may be provided by the staining of CD34 (29,30). Similarly to these reports, in our study, all specimens were stained positively with CD34 and the intensity of staining was ranging from (+) to (+++). However, the expression level was not found significant statistically between stage II and III tumors ($p>0.05$), and the intensity of immunostaining with CD34 was found as a significant prognostic factor to predict the survival in stage II and III tumors.

Finally, although HER2/neu is among EGFR family, in our study none of our germ cell tumor specimens were stained positively with HER2/neu by immunohistochemistry. The role of other subtypes of EGFR family in predicting the survival of germ cell tumors needs to be investigated.

In conclusion, our study revealed that MIB-1, HER2/neu or CD34 are not useful as a prognosticator to predict the survival in patients with stage II and III testicular germ cell tumors. The intensity of CD34 and MIB-1 immunostaining increased with advancing stage. The role of these markers in clinical stage and prognostic evaluation needs further investigation.

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