C-Erb-2 Gene Amplification and Chromosomal Anomalies in Bladder Cancer: Preliminary Results

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Studies recently suggested that different genetic factors are involved in the development and progression of bladder cancer. In this study, 30 consecutive patients affected by bladder neoplasm were evaluated in order to analyze the frequency of c-erb-2 gene amplification and chromosome 7, 9, 17 aneusomy using fluorescence *in situ* hybridization (FISH) technique. C-erb-2 gene amplification, chromosome 17 gain and aneusomy were respectively observed in 3.7% (1/27), in 47% (12/27) and in 74% (20/27) of examined tumors. Moreover, chromosome 7 and 9 aneusomy were detected in 74% (20/27) and in 72% (16/27) of specimens. A statistically significant correlation was observed between chromosome 17 aneusomy and tumor stage and grade (r: 0.642, p=0.0001; r: 0.385, p=0.04, respectively). In conclusion, we observed a low incidence of C-erb-2 gene amplification, while chromosome 17 aneusomy was confirmed as a marker of advanced and aggressive bladder cancer.

Key Words: Bladder cancer, Oncogenes, HER-2/neu, c-erB-2, Aneusomy

Bladder cancer ranks fourth overall in the number of newly diagnosed cancers and tenth in causes of cancer deaths (1). Transitional cell carcinoma (TCC) results in more than 90% of all cases of bladder cancer with most of the remainder being squamous cell carcinoma (5%), adenocarcinoma (2%) or rhabdomyosarcoma (1%). Thirty percent of bladder tumors are initially present as muscle invasive or metastatic disease. Of these, 50% will die 2-3 years after the diagnosis despite aggressive local therapy. On the other hand, 70% of TCC cancers are "superficial" at initial presentation. These tumors are confined to the mucosa (70%) or to the lamina propria (30%). Approximately 50-70% of TCC often recur: 10-30% showing higher grade and stage progression (2). The standard treatment for superficial transitional cell carcinoma (STCC) is transurethral resection (TUR) followed by, in high grade lesions, an intravesical therapy (chemotherapy or immunotherapy).

Although different factors have been correlated with patient outcome, initial histological stage, multifocality and grade were considered the most important factors influencing the clinical and surgical management of this kind of neoplasm (1,2). Development of bladder cancer was generally accepted as a consequence of genetic instability and involvement of chromosomes 1, 7, 9 and 17 in the uroepithelial oncogenesis as a frequent event (3,4). In the regions of the DNA gains, high level amplification was found at 17q11-21.3 bands in primary and metastatic tumor and it has been shown that the HER-2 protooncogene (also known as c-erb 2 or neu) was located at this locus (5, 6). Human Her 2/neu (c-erb-2) proto-oncogene encodes a Mr 185,000 transmembrane tyrosine kinase receptor. The c-erb-2 protein is a growth factor receptor with the ability to stimulate cell growth. To date, gene amplification and protein expression of c-erb 2 have been reported in different adenocarcinomas, including some gastric cancers, breast cancers (7,8) and many other tumors. Although its specific ligand has not been identified, c-erb-2 has been found to be important for the regulation of cell growth and differentiation. Several studies concerning the incidence of c-erb-2 amplification in bladder cancer have been performed but with conflicting results (9-12). Furthermore, the relationship between c-erb-2 gene amplification and the development and progression of bladder cancer is still debated. The clinical significance of c-erb gene amplification remains controversial. Until now the usual method to test for c-erb-2 has been

immunohistochemical tissue staining. However, several authors have recently described the use of the fluorescence *in situ* hybridisation (FISH) method for the analysis of c-erb-2 gene amplification (12,13). Using the FISH technique, different reports have also showed the involvement of chromosome 7, 9, 17 in bladder carcinogenesis (14-16). To our knowledge, there were few reports evaluating c-erb-2 amplification and at the same time the presence of chromosome aneusomy in bladder cancer. Aim of our study was to analyze by FISH the frequency of c-erb-2 gene amplification and aneusomy of chromosome involved in bladder carcinogenesis.

Materials and Methods

Source of tumor specimens. The study included 30 male patients (mean age 67 years, median 65, range 45-80) with bladder neoplasm. Specimens from a total of 30 consecutive cases of clinically diagnosed bladder cancer, 15 primitive and 15 recurrences, were obtained by transurethral resection. The diagnosis, classification and tumor grading were based on light microscopy examination using the criteria of the World Health Organization (WHO) classification of uroepithelial tumors (17). Staging was performed in accordance with the UICC tumor-node metastasis (TNM) system (18). All classifications were done without prior knowledge of cytogenetic findings. All samples were reviewed by one pathologist with experience in uropathology. Informed consent was obtained from all patients. Fourteen tumors were confined to the bladder mucosa (Ta), 8 showed minimal invasion limited to the lamina propria (T1), 5 showed invasion of the tunica muscolare (T2), 1 had papillary hyperplasia and in two patients histology report showed normal urothelium. All the samples were rapidly frozen and stored at -80C° before FISH analysis.

FISH analysis. All cytogenetic studies were done on cytological imprints of the resected material and gently pressed onto the surface of sialinized slides. Touch preparations (imprints) were spray fixed immediately after collection.

Probes. The chromosome enumeration probes are pericentromeric fluorescent-labeled for use in the FISH assay (Vysis, Inc.) specific for the centromeric region of chromosome 7 (D7Z1), 9 (D9Z5) and 17 (D17Z1). The probe for HER-2 evaluation (Path Vysion HER-2 DNA probe Kit; Vysis, Downers Grove, IL, USA) is a fluorescent labeled nucleic fragment for use in the FISH assay on tissue sections fixed on slides. The LSI HER-2 (Spectrum Orange) probe contains DNA sequences specific for the c-erb-2 human gene locus, and hybridizes to the 17q11.2-q12 region of human chromosome 17. The chromosome 17 enumeration probe (Spectrum Green) was used as control to determine copy number of chromosome 17 when the c-erb-2 copy numbers were counted. The ratios of average copy numbers per cell were calculated to establish the presence of amplified c-erb-2. After aging, procedure "Hybrite System" (Vysis) was used. Hybrite is an open system for hands-free 73°C denaturation and hybridization when using *in situ* DNA probe procedures. After overnight hybridization at 37°C, post-hybridization wash was applied using 0.4xSSC/NP40 at 73°C. The slides were then counterstained using DAPI (4,6-diamidino-2-phenylindole) and processed with an Olympus BX60 fluorescence microscope equipped with a 100 watt mercury lamp. Separate band pass filters were used for the detection of the CEP1 and CEP 7 probe signals (Spectrum Orange) and for the detection of the CEP 9 and CEP 17 probe signals (Spectrum green). After DAPI counterstaining, fluorochrome signals were captured individually and images were generated via computer with Quips Genetic Workstation and Imaging Software (Vysis). The slides were observed at 100x magnification. A minimum of 100 well-defined nuclei were scored for each hybridization. Clumps and overlapping nuclei were disregarded. Only nuclei with unambiguous chromosome centromeric hybridization were scored. Nuclei with obvious cross-hybridization and/or high background were not scored. The amplification is defined as a HER-2 to CEP 17 ratio greater than 2.

Statistical Analysis. The cutoff level for aneusomy in imprints was established beforehand at 20% for chromosomes 1, 7, 9 and 17. Our statistical evaluation was based on the mean percentage of in toto aneusomic cells. T-test was used for statistical analysis. Association between histological classification and chromosomal aberrations was measured using Pearson correlation coefficient. $p < 0.05$ was considered statistically significant.

Results

Touch preparations were obtained from 30 patients with uroepithelial carcinoma. For 3 patients the FISH assay was not available. Histology report, C-erb gene

Table I - Histological diagnosis of tissue samples taken from 27 patients undergone transurethral resection and results of the genetic analysis using FISH (A: aneusomic, D: disomic)

N°	Grade	Stage	C-erb-2 Amplification	Gain of Chr 17	C-erb-2/CEP 17 ratio	Chr 7	Chr 9	Chr 17
1	G2	Ta	no	-	0.92	A	A	A
2	G2	Ta	no	-	0.85	A	A	A
3	G2	T1	no	+	0.89	A	A	A
4	G2	T2	no	+	1.05	A	A	A
5	G2	Ta	no	+	1.05	A	A	A
6	G0	T0	no	-	1.30	A	D	D
7	G3	T1	no	+	0.76	A	A	A
8	G1	T1	no	+	1.07	D	D	A
9	G2	Ta	no	-	1.04	A	A	A
10	G2	T1	no	+	1.05	A	A	A
11	G2	T2	no	+	0.87	A	A	A
12	G2	Ta	no	-	1.13	D	A	D
13	G1	Ta	no	+	0.91	A	A	A
14	G2	T1	no	-	0.91	D	D	D
15	G1	Ta	no	-	0.81	A	D	D
16	G2	T2	no	+	1.20	A	A	A
17	G3	T1	no	-	1.32	A	A	A
18	G2	Ta	no	-	0.96	D	A	D
19	G2	Ta	no	-	0.97	D	D	D
20	G1	Ta	no	-	1.05	A	A	A
21	G2	Ta	++	+	3	A	A	A
22	G3	T2	no	+	1.86	A	A	A
23	G2	T1	no	-	1.12	D	A	D
24	G0	T0	no	-	1.04	A	A	D
25	G2	T2	no	+	1.05	A	A	A
26	G2	Ta	no	+	1.60	A	A	A
27	G2	Ta	no	-	1.05	D	D	A

amplification, gain of chromosome 17, c-erb/CEP 17 ratio and 7, 9 ,17 aneusomies of 27 patients object of our study are shown in Table I. As illustrated in Table II, C-erb2 gene amplification was found in 3.7% (1/27) of all tumors. Gain of chromosome 17 was observed in 47% (12/27) of patients. It was identified in 100% (5/5) of patients with high stage (T2) and in 31% (7/22) (p=0.0001) of patients with low stage (≤T1). Aneusomy of chromosome 17 was observed in 74% of patients (20/27). In particular, aneusomy of chromosome 17 was observed in 100% (5/5) of patients with T2 bladder cancer and in 69% (15/22) of patients with low stage (p= 0.005).

As far as chromosome 7 is concerned, aneusomy was observed in 74% of patients (20/27): in particular, in 100% (5/5) of patients with T2 bladder cancer and in 68% (15/22) of patients with low stage (p=0.005). Moreover, we detected chromosome 9 aneusomy in 77% of patients (21/27): this alteration was observed in 100% (5/5) of patients with T2 bladder cancer and in 72% (16/22) of patients with low stage (p=0.01). When we correlated histological variables with evaluated chromosome aneusomy, we obtained a statistically correlation between chromosome 17 aneusomy, stage and grade (r:0.642, p=0.001 and r:0.385, p=0.04, respectively). No statistically significant correlation was

Table II - Relation between FISH results and stage in bladder cancers

	No. of cases		
	≤ T1	T2	p
C-erb-2 gene amplification	1 of 22	0 of 5	0.32
Gain of Chromosome 17	7 of 22	5 of 5	0.0001
Aneusomy of Chromosome 17	15 of 22	5 of 5	0.005
Aneusomy of Chromosome 7	15 of 22	5 of 5	0.005
Aneusomy of Chromosome 9	16 of 22	5 of 5	0.01

Table III - Correlation between histological classification and chromosomal aberrations

	STAGE		GRADE	
	p	r*	p	r*
Chr. 7 aneusomy	0.07	0.350	0.089	0.334
Chr. 9 aneusomy	0.139	0.292	0.07	0.350
Chr. 17 aneusomy	0.001	0.642	0.04	0.385

**(Pearson correlation coefficient).*

observed between tumor grade, stage and chromosome 7, 9 aneusomy, as demonstrated in Table III. No statistically significant differences (p= 0.775) were observed between aneusomy values (mean percentage) of chromosome 7, 9 and 17 (data not showed).

Discussion

C-erb-2 gene, the human homologue of the rat neu gene, code for a transmembrane protein having tyrosine kinase activity (19). Recent data suggested that c-erb-2 played a role in tumor progression by promoting the invasive capacity of tumor cell through increased cell adhesion, increased cell motility and up-regulation of proteolytic enzymes. C-erb-2 has been extensively studied in breast cancer, where it has been demonstrated that protein overexpression, which was generally, but not always, due to gene amplification, inversely correlated with estrogen receptor and was associated with a poor prognosis (20). Recently, studies indicated that c-erb-2 protein was expressed by a significant proportion of transitional cell tumors of the bladder. However, reports of c-erb-2 overexpression in urothelial carcinoma ranged from 2 to 74% and its clinical and prognostic significance are still debated (9-12,19). Differences in incidence could be related to different methodologies used for the assessment of the c-erb-2 status as the polymerase chain reaction technique (PCR), the immunohistochemistry (IHC) or FISH analysis. Our study assessed c-erb-2 gene amplification by FISH analysis, which is the method recently used for testing Her2-status breast cancer. Cianciulli et al. (13) and Pauletti et al. (21), comparing FISH and IHC for the evaluation of HER-2 in breast cancer, showed that FISH analysis, performed with a probe approved by the U.S. Food and Drug Administration for Her-2 oncogene (Vysis), should be the best test to compare the results between different clinical studies, since this method should be less technique dependent than IHC. In our study, c-erb-2 gene amplification was only identified in 3.7% of all tumors. This data seem to be similar to those obtained by other authors using similar techniques. Sauter et al. (22), using FISH analysis, reported a 7% c-erb-2 gene amplification and observed that it was more frequent in high stage tumor. Ochta et al. (12) also observed a low incidence of c-erb-2 gene amplification in a series of 29 bladder tumors. These data, obtained by FISH analysis, were also comparable with those obtained by Mellon et al. (10) who observed a 4% incidence of c-erb-2 gene amplification but using Southern blotting analysis. However, different studies evaluating in particular high stage bladder cancer and using the PCR or IHC technique showed higher incidence of c-erb-2 gene amplification (23-24). These data seem to underline that c-erb-2 oncogene was involved in bladder cancer tumorigenesis but there was no evidence of high gene amplification in the early phase of tumor growth. It was assumable that further studies using FISH analysis and evaluating high stage bladder cancer should elucidate the role of this oncogene in bladder tumorigenesis. Using FISH, numerical change of chromosome 7, 9 an 17 were also analysed. Waldam et al. (14) suggested a

direct correlation between an increased number of chromosome 7 and aggressive tumor behavior. Changes in chromosome 9, where a tumor suppressor gene was mapped to the 9p21 region, appeared to be responsible for superficial bladder tumor (15). Aneusomy of chromosomes 7 and 9, should represent an intermediate biomarker of bladder tumorigenesis (3). Li et al. (16) demonstrated that the percentage of hiperdiploid cells for chromosome 17 was correlated with p53 overexpression, increasing tumor grade and advanced pathologic stage. These authors pointed out that the occurrence and extent of numeric aberrations of chromosome 17 should be associated with the evolution of aggressive growth. In our study, we observed numerical aberrations of chromosomes 7, 9 and 17 with similar patterns in all analyzed specimens. In particular, gain of chromosome 17 and aneusomy of chromosome 7, 9 were observed in 100% of patients with high stage bladder cancer. Regarding chromosome 17 aneusomy, cytogenetic findings revealed an accumulation of chromosomal aberrations during bladder progression, showing a significative correlation with histopathological classification. These results were consistent with the results reported by other studies (3,22) and suggested that chromosome 17 aneusomy was strongly associated with aggressive and advanced bladder cancer. Our data seems to confirm the involvement of chromosome 7, 9 and 17 abnormalities in the development and progression of bladder cancer. A correct assessment of chromosome 7, 9 and 17 aneusomy should be used in the evaluation of patients with bladder cancer in order to identify patients prone to an aggressive disease who should benefit from preventive interventions. Chromosome 17 aneusomy should be applicable as a tumor marker for the evaluation of malignant potential, invasiveness and prognosis of human bladder cancer. Our study demonstrated the low incidence of C-erb-2 gene amplification on transitional cell carcinoma. Chromosome 17 aneusomy was confirmed to be a marker of advanced and aggressive bladder cancer.

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