

Expression of HLA-G in Cancer Bladder

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This study comprised 42 patients with cancer bladder, who underwent radical cystectomy. The aim of work is to determine if HLA-G is expressed on tumor cells, derived from cancer bladder. We studied HLA-G-mRNA expression using RT-PCR and HLA-G cell surface expression by immunohistochemistry (IHC) staining technique. HLA-G was expressed in 28.6 % of cancer cases as determined by PCR and on 16.7% of cases determined by IHC staining. The sensitivity, specificity and accuracy of IHC were 58.3%, 96% and 81.7% respectively as compared to PCR results. There was a highly significant increase in the expression of HLA-G on cancer bladder cases with metastatic prostate infiltration (P= 0.021). It is concluded that HLA-G is ectopically expressed on cancer bladder malignant cells both at molecular and protein levels. However, it is not significantly associated with histologic type, tumor grade, stage, T category, schistosomiasis and lymph node involvement.

HLA-G is a non classical MHC class I molecule. It is selectively expressed by extravillous trophoblast in the human placenta, a tissue that does not express HLA A or B molecules (King et al., 2000). It is tempting to propose that tumor cells resemble this unique HLA class I phenotype as they frequently lose classical HLA. A, B and C class I expression. Such peculiar HLA class I distribution would in theory allow tumor cells to escape from T and NK cell cytotoxicity (Real et al., 1999). HLA-G molecule is characterized by a transcription of spliced messenger RNAs encoding for at least four membrane-bound and two soluble HLA-G isoforms (Carosella et al., 1999).

HLA-G class I b gene appears to be a functional locus, 1) HLA-G is capable of presenting nonamer peptides and of exerting antigen-presenting functions 2) HLA-G is a ligand for three natural killer (NK) and immunoglobulin-like receptor-1/ immunoglobulin like transcript ILT-2, ILT-4 and p49, 3) HLA-G proteins have been detected in endothelial cells of placental chorionic villi,

as well as in amniotic fluid and in some medullary thymic epithelial cells, 4). In addition to the classical MHC class I roles (antigen presentation and ligation to NK receptors inducing inhibitory and/or activatory signals), HLA-G exert other novel functions: first, HLA-G was involved in the control of HLA-E expression by furnishing the appropriate class I leader sequence nonamer peptide, second, HLA-G could be a regulator of placental angiogenesis, third, soluble HLA-G isoforms may act as specific immunosuppressors during pregnancy (Le Bouteiller and Blaschitz, 1999).

In contrast to the classical HLA class Ia molecules, the nonclassical HLA-G primary transcript is alternatively spliced to generate several mRNAs that encode four membrane-bound proteins (HLA-G1 to G-4) and three soluble proteins (HLA-G5 to G7) (Park et al., 2004). Differential expression of mRNA transcripts corresponding to soluble and memberane-bound HLA-G was observed in some tumor biopsies. HLA-G protein expression was detected in tumors that

exhibited high levels of HLA-G transcription. Tumors expressed high levels of HLA-G provides additional clues as to how a tumor can be selected to escape from cytotoxic antitumor responses (Paul et al., 1999).

Today tumor is considered to result from a multistep genetic alterations. The details of which are still controversial. The urothelium can be transformed into one of three histologically neoplastic states: low-grade papillary transitional cell carcinoma (TCC), carcinoma *in situ* and associated flat, high grade, invasive TCC, and squamous cell carcinoma. Squamous cell carcinoma which is relatively, uncommon in the United States, has a distinct cause and is frequently associated with schistosomiasis (Jones and Ross, 1999). Bladder cancer is the fifth most common cause of cancer deaths in men. Bladder cancer accounts for 2.6% of all cancer deaths in men and 1.4% in women. (Messing and Catalona, 1999). Since the 1950s, the incidence of bladder cancer has risen for an overall increase of approximately 50% (Annual Cancer Statistics Review, 1987). Bladder cancer is nearly three times more common among men than women. In men, it is the fourth most common cancer after prostate, lung, and colorectal cancers and is accounting for 5.5% of all cancer cases (Boring et al., 1995). In women, it is the eight most common cancer, accounting for 2.3 % of all cancers.

NK recognition of carcinomas is not clear. Compared with normal cells, many tumor cells expressed low MHC class I levels and are thus potentially susceptible to NK cell attack (Bernards et al., 1989, Smith et al., 1989, Schrier and Petenburg 1993, Singal and Sinder, 1996). Most studies indicated that HLA class I molecules protect human melanoma cells from NK mediated cytotoxicity. Confluent carcinoma cells, rapidly growing carcinoma cells expressed more HLA class I and were relatively resistant to NK mediated cytotoxicity (Quillet et al., 1988). A full

understanding of how NK cells control SCC requires characterization of the NK receptors and HLA ligands that inhibit NK cytotoxicity, migration and cytokine secretion. A number of stimulatory NK receptors and ligands have been identified. Although not all stimulatory ligands are known, it is likely that target cells with insufficient stimulatory ligand expression will not activate NK cells, even when interactions between inhibitory NK receptors (KIRs) and MHC class I ligands are prohibited (Lanier et al., 1997). In contrast high target cell stimulatory ligand expression may strongly activate NK cells, potentially overriding abundant inhibitory NK receptor / MHC class I interactions.

It has been found that class I expression is commonly lost or diminished in prostate cancer (Bander et al., 1997). It seems clear that MHC antigen expression on tumor cells is important in triggering the immune response by autologous lymphocytes. A deficiency in or lack of MHC class I antigens may have profound effects on T and NK cell activity. In experimental models, variation in the expression of MHC class I antigens have been shown to exert a decisive influence on local tumor growth and metastasis.

There is no doubt that we need to learn more about how to manipulate the expression of MHC class I and II antigens in human tumors, in order to stimulate the immune response. Metastatic prostate cancer cells can escape T cell recognition via divergent mechanisms of defective class I assembly (Sanda et al., 1995). The specific underexpression of TAP-2 gene product provides evidence for a regulatory pathway controlling TAP-2 gene expression in human cancers that may not affect class I MHC-heavy chain expression. CD8 MHC-class I dependent CTL present in urothelial carcinomas are functional and may participate in the anti-tumor immune response (Housseau et al., 1997). The functions of CD8 in the TCR complex are thought to be

signaling. CD8 can bind to HLA-G, therefore, it is possible that a cell bearing CD8 may interact with HLA-G expressing cells (Sanders et al., 1991).

The aim of this study is to determine if HLA-G is expressed on tumor cells derived from cancer bladder.

Materials and Methods

This study comprised 42 patients with cancer bladder, admitted to the Urology and Nephrology Center, Mansoura University. They were 29 males and 13 females, their age ranged from 41-68 years. They underwent radical cystectomy.

They were histologically diagnosed as 23 TCC (54.5%) and 19 SCC (45.2%), grades from (G1-G3) and stage (I-IV).

Specimens

Fresh frozen and paraffin sections from cancer bladder surrounded by normal tissues, were submitted to the following:

- Total RNA extraction.
- RT-PCR.
- Immunohistochemical staining technique.

Fetal membranes of human placenta consist of amniotic and chorionic membranes were used as a positive control (expressing HLA-G)

Sample collection and storage

Cancer bladder specimens, part of normal tissues surrounding the tumor and the two membranes of the placenta (are gently peeled apart, 2mm² pieces of chorionic were excised with a scalpel) and all specimens were snap frozen in a liquid-nitrogen cooled isopentane dry shipper (Taylor-Wharton, Theodore, AL) until analysis. During tissue disruption for RNA isolation, it is crucial that the denaturant RNase zap (Ambion), used to render RNases inactive, be in contact with the cellular contents at the moment that the cells are disrupted. The samples must then be ground with a tissue TeaRor (Biospec products, Inc) into a fine particles.

The total RNA was extracted from 2 mg of tissue and homogenized in RNA **now** (BIOGENTEX): 1ml/100 mg tissue.

Yield and quality of RNA extraction

The RNA amount is evaluated by measuring the OD₂₆₀ of a 1:250 dilution in water (1 OD₂₆₀ = 40 µg of RNA/ml), A₂₆₀ /A₂₈₀ should be > 1.6. The quality of

RNA was checked by minigel electrophoresis in a 1.5% agarose and visualized by ethidium bromide staining. After Electrophoretic migration, gels were documented with polaroid pictures on an ultraviolet transilluminator. Three bands corresponding to the total RNAs (28S, 18S, 5S) should be observed. A positive control was consistently positive in all PCR amplifications, whereas negative controls without complementary DNA were consistently negative.

Reverse transcriptase-polymerase chain reaction (RT-PCR) Analysis

cDNAs were prepared from 5 µg of total RNA, by using oligo-(dT) primer (pharmacia Biotech) and Molony Murine Leukaemia virus transcriptase: M-MLV- Rt (Life Technologies). PCR amplifications (DNA thermal cycler, progene, pharmacia) were performed using exon 2-specific primer: (pan HLA-G primers) G275 (5'-GGAAGAGGAGA CACGGAACA-3') (Kirszenbaum et al., 1994), and G1004 R (5'-CCTTTTCAATCTGAGCT CTTCTTT-3'), HLA-G5 specific primers: exon 3-specific primer G.526 (5'-CCAATGTGGCTGAACAAAGG-3'), (Kirszenbaum et al., 1994), and intron 4-specific primer G.i4b (5'-AAAGGAGGTGAAGGTGAGGG-3') (Moreau et al., 1995).

PCR conditions used are as follows: 94°C for 1 min (G.257), or 61°C (G.257/G. 1004R, G526/G.i4b) for 1 min 30 sec, 72°C for 2 min (35 cycles), and the last extension step at 72°C was prolonged to 7 min. Coamplification of β-actin cDNA was carried out with β amplimer sets (clontech) for 16 cycles to evaluate comparative amounts of cDNA in samples. Absence of contaminant DNA was controlled by concomitant amplification of the PCR mixture without a template (H₂O).

Immunohistochemical staining technique

Fresh frozen and paraffin sections undergone Immunohistochemical staining (DaKo), using monoclonal antibodies: 87G (in a dilution 1/100 in PBSA) for HLAG1 & G5, 4H84 (in a dilution 1/500 in PBSA) for IgG1 specific antibody), TP 25.99 (in a dilution 1/10 in PBSA) for HLAA, B, C, E, and mouse IgG 2a (in a dilution 1/100 in PBSA as negative control antibody). Monoclonal antibodies supplied kindly from E.D Carosella, Hospital Saint Louis, Paris.

Statistical analysis

Statistical analysis was done by using SPSS statistical package for social science program version 10, 1999. The quantitative data were presented in the form of means and standard error of mean and range. Student T test was used for quantitative data. The qualitative data were presented in the form of number and percentage.

Chi-square test was used for qualitative data. P significance is < 0.05 . Sensitivity, specificity and accuracy were calculated for IHC results according to PCR results.

Results

In this study we evaluated 42 cancer bladder specimens (23 TCC and 19 SCC of various stages (I-IV) and grades (G1-G3) as shown in table (1). The lymph nodes were free in 31

patients (73.2 %) and infiltrated in 11 patients (26.8 %).

There was no significant difference in HLA-G gene expression in PCR positive and PCR negative cases as regard the age and sex of patients, tumor grades, tumor stage, histological types (TCC or SCC), presence or absence of bilharzial ova, T category, and lymph node involvement.

Table 1. Demographic and clinical data of cancer bladder patients Characteristics of the studied group.

Age		
Mean \pm SE		59.9 \pm 1.31
Range		37 – 68 years
Sex		
	Number	%
Male	29	69
Females	13	31
Total	42	100
Tumor grade		
G1	12	28.6
G2	18	42.9
G3	12	28.6
Total	42	100
Tumor stage		
I	6	14.3
II	8	19.0
III	15	35.7
IV	13	31.0
Total	42	100
Histologic type		
TCC	23	54.5
SCC	19	45.2
Total	42	100
Bilharzial ova		
Present	27	64.3
Absent	15	35.7
Total	42	100
T category		
PT2	15	35.7
PT3	23	54.8
PT4	4	9.5
Total	42	100
L.N involvement		
+ve	11	26.8
-ve	31	73.2
Total	42	100

There was highly significant expression of HLA-G gene in bladder cancer patients with prostatic infiltration, P 0.021. As shown in table (2) As regard HLA-G protein cell surface expression by IHC staining, there was no significant difference between positively and negatively stained specimens in tumor grades and stages, TCC or SCC, presence or absence of bilharzial ova, T category, lymph

node involvement and prostatic infiltration as shown in table (3).

Comparative study between PCR and IHC staining techniques showed that, the sensitivity of IHC results was 58.3 %, specificity was 96% and accuracy was 81.7 % in relation to PCR results as shown in table (4).

Table 2. HLAG-mRNA expression in patients with cancer bladder PCR results in relation to studied group characteristics

	HLAG-mRNA expression*				Test of signification
	Positive (n=12)		Negative (n=30)		
Age					
Mean \pm SE	56.08 \pm 2.91		53.1 \pm 1.13		P= 0.31
Range	41 - 68 ys.		37 - 68 ys.		
Sex	Number	%	Number	%	P= 0.091
Male	6	20.7	23	79.3	
Female	6	46.2	7	53.8	
Tumor grading					P= 0.063
G1	4	33.3	8	66.7	
G2	2	11.1	16	88.9	
G3	6	50.0	6	50.0	
Tumor stage					P= 0.77
I	1	16.7	5	83.3	
II	2	25.0	6	75.0	
III	4	26.7	11	73.3	
IV	5	38.5	8	61.5	
Histologic type					P= 0.32
TCC	8	34.3	15	65.2	
SCC	4	21.1	15	78.9	
Bilharzial ova					P= 0.35
Present	9	33.3	18	66.7	
Absent	3	20.0	12	80.0	
T category					P= 0.47
PT2	3	20.0	12	80.0	
PT3	7	30.4	16	19.0	
PT4	2	50.0	2	50.0	
L.N involvement					P= 0.46
+ve	5	35.7	21	75.0	
-ve	7	25.0	9	64.5	
Prostate					P= 0.021
Infiltration	2	100	--	--	
Free	10	25.0	30	75.0	

*By PCR

Table 3. HLAG cell surface expression in patients without cancer bladder.

	HLAG cell surface expression*				P=
	Positive (n=7)		Negative (n=35)		
Age					
Mean ± SE	56.25 ± 3.88		53.48 ± 1.39		0.79
Range	41 – 68 ys.		37 – 68 ys.		
Sex	Number	%	Number	%	
Male	4	13.8	25	86.2	0.45
Female	3	23.1	10	76.9	
Tumor grading					
G1	2	16.7	10	83.3	0.13
G2	1	5.6	17	94.4	
G3	4	33.3	8	66.7	
Tumor stage					
I	--	--	6	100	0.21
II	2	25.0	6	75.0	
III	1	6.7	14	93.3	
IV	5	30.8	9	69.2	
Histologic type					
TCC	5	21.7	18	78.3	0.33
SCC	2	10.5	17	69.2	
Bilharzial ova					
Present	5	18.5	22	86.7	0.66
Absent	2	13.3	13	81.5	
T category					
PT2	3	20.0	12	80.0	0.76
PT3	3	13.0	20	87.0	
PT4	1	25.0	3	75.0	
L.N involvement					
+ve	4	28.6	10	71.4	0.14
-ve	3	10.7	25	89.3	
Prostate					
Infiltration	1	50.0	1	50.0	0.19
Free	6	15	34	85.0	

*by IHC

Table 4. Sensitivity, specificity and accuracy of the immunohistochemistry technique based on using PCR as a gold standard.

IHC	PCR				No
	+ve		-ve		
	No	%	No	%	
+ve	6	85.7	1	14.3	7
-ve	6	17.1	29	82.9	35
Total	12	28.6	30	71.4	42

Sensitivity of IHC 58.3%

Specificity of IHC 96%

Accuracy of IHC 83.3%

Discussion

In this study, the TCC was reported in 54.5 % cases underwent radical cystectomy, while SCC reported in 45.2 % of cases and bilharziasis was present in 64.3% of cases. Rafla et al. (1994) and Ghoneim et al. (1997) reported that a high incidence of bladder cancer is observed in Egypt and some other parts of Africa and the middle East (20.6 % of all malignant neoplasms). It is almost always associated with schistosomiasis. In contrast to Western countries, 65% to 77 % are SCC and 22 % are TCC (Pycha et al., 1999). In Europe and North America, TCC of the urinary bladder is the fifth most common cancer. Approximately 80% of the tumors are diagnosed as superficial tumors and only 10% to 20% are invasive or metastatic carcinomas at the time of diagnosis (Sandbrg and Berger, 1994). Squamous cell carcinoma (SCC) makes up 7 %, it is usually associated with chronic infection or irritation of the transitional epithelium and is considered to have a poorer prognosis (El-Bolkainy et al., 1981).

In this study, HLA-G was observed in some tumor biopsies. HLA-G protein expression was detected in tumors that exhibited high levels of HLA-G transcription. This provides additional clues as to how a tumor can be selected to escape from cytotoxic antitumor response (Paul et al., 1999). In our study HLA-G-mRNA was expressed in 28.6 % of cancer bladder specimen determined by IHC staining technique.

HLA-G has not been expressed on normal tissues surrounding the tumors. Pan gault et al. (1999) demonstrated that healthy tissues surrounding the tumor tissue do not express HLA-G molecules. On the other hand HLA-G products were detected in activated macrophages and dendritic cells localized in tumoral biopsies derived from lung, ovary,

larynx, liver, colon, breast and kidney tissues *exvivo* and are not expressed in the malignant transformed cells. These observations demonstrate that HLA-G is not a marker of malignant cells but appear as a gene expressed in tumor-associated macrophages and dendritic cells.

Bladder cancer can spread locally to invade adjacent organs, including the prostate, uterus, vagina, ureters, rectum and intestine. More than 40% of men undergoing cyctectomy for muscle-invasive bladder cancer have involvement of the prostate (Wischnow and Ro, 1988). In our study 4.8 % of cases have metastatic cancer prostate and HLA-G was highly significantly expressed in those cases (P= 0.021). In conclusion, HLA-G is ectopically expressed on cancer bladder malignant cells both at molecular and protein levels.

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