Immunogenetics (2001) 53:606–610
DOI 10.1007/s002510100371

BRIEF COMMUNICATION

**Pilar Jiménez · Teresa Cabrera · Rosa Méndez
Clara Esparza · José Manuel Cozar · Miguel Tallada
Miguel A. López-Nevot · Francisco Ruiz-Cabello
Federico Garrido**

A nucleotide insertion in exon 4 is responsible for the absence of expression of an *HLA-A*0301* allele in a prostate carcinoma cell line

Received: 11 June 2001 / Revised: 15 August 2001 / Published online: 25 September 2001

Keywords HLA · Null allele · Mutation · Microsatellite instability · Tumor

Serological typing methods have been used traditionally to define the polymorphism of HLA antigens. However, since the introduction of DNA typing methods, discrepancies between serological and molecular methods have been reported. Thus, certain alleles type easily at the DNA level but lack cell surface expression and are, therefore, undetectable by serological typing. These discrepancies arise from structural differences between the expressed and the deficient forms of the gene (Parham 1997). In this report, based on a combination of cellular and molecular methods, we describe a nucleotide insertion responsible for the loss of expression of an *HLA-A*0301* allele in a prostate carcinoma cell line.

DU145 is a tumor cell line derived from a cerebral metastasis of a prostate carcinoma. The cell line was obtained from the American Type Culture Collection (ATCC; Rockville, Md.). Molecular typing for *HLA* class I (*A* and *B* loci) was performed on genomic DNA from the cell line using the *HLA-A* and *HLA-B* sequencing-based typing kits and ABI PRISM 377 DNA Match/Maker sequencer allele identification software (Applied Biosystems, Foster City, Calif.). The *HLA* genotype from the DU145 cell line (sequences from exons 2 and 3) was *HLA-A*0301*, *A*3303*/*HLA-B*5001*, *B*5701*. HLA class I expression on the cell surface was measured by indirect immunofluorescence using the appropriate anti-class I monoclonal antibody (mAb) and fluorescein isothiocyanate-labeled rabbit anti-mouse Ig $F(ab)_2$ fragments (Sigma, St Louis, Mo.). Fluorescence was analyzed with a FACsort flow cytometer (Becton-Dickinson, Mountain View, Calif.) and showed loss of expression of the *HLA-A*0301* allele (Fig. 1). This finding was confirmed using two different mAbs that specifically recognize this antigen (mAb 160.30 and 361.1, kindly provided by K. Gelsthorpe, Sheffield BTS, UK). Because interferon-γ upregulates HLA expression in several cell types, we also examined the effect of this cytokine. Cells were treated with human recombinant interferon-γ (800 units/ml) (Amersham, Little Chalfont, UK) for 48 h and cell surface HLA class I expression was then determined by immunofluorescence as described previously. In DU145 cells, all expressed alleles were upregulated, whereas HLA-A3 remained undetectable (Fig. 1).

To determine whether the lack of *HLA-A*0301* expression of in DU 145 cells was due to failure of transcription, RT-PCR analysis was done using specific primers for the *HLA-A* locus. Amplification was performed using 2 µl of cDNA and the locus-specific primers A-S and A-AS (Table 1). Primers derived from exon 2 and exon 5 were used to amplify a 790-bp fragment. Sequence analysis of this product, using dye terminators and the ABI-PRISM 377 DNA Sequencer (PE Applied Biosystem), revealed the absence of *HLA-A*0301* transcript. Because single point mutations within the 5′ untranslated region have been observed to silence HLA class I expression (Lardy et al. 1992), we explored the promoter region of the *HLA-A3* gene by specific amplification followed by direct sequencing of genomic DNA. The *HLA-A* locus-specific primer Pra was matched with the allele-specific primer for *HLA-A3* A3-AS (Table 1). This pair of primers amplified an 810-bp fragment that spanned the region from position –385 upstream of the first ATG to nucleotide 224 of exon 2. Sequencing the PCR product showed the upstream regulatory region to contain the correct genomic sequence (data not shown).

P. Jiménez · T. Cabrera · R. Méndez · C. Esparza
M.A. López-Nevot · F. Ruiz-Cabello · F. Garrido (✉)
Servicio de Analisis Clinicos,
Hospital Universitario Virgen de las Nieves,
Avda. Fuerzas Armadas s/n, 18014 Granada, Spain
e-mail: fgarrido@hvn.sas.cica.es
Fax: +34-958-283147

J.M. Cozar · M. Tallada
Servicio de Urología,
Hospital Universitario Virgen de las Nieves,
Universidad de Granada, Avda. de las Fuerzas Armadas s/n,
18014 Granada, Spain

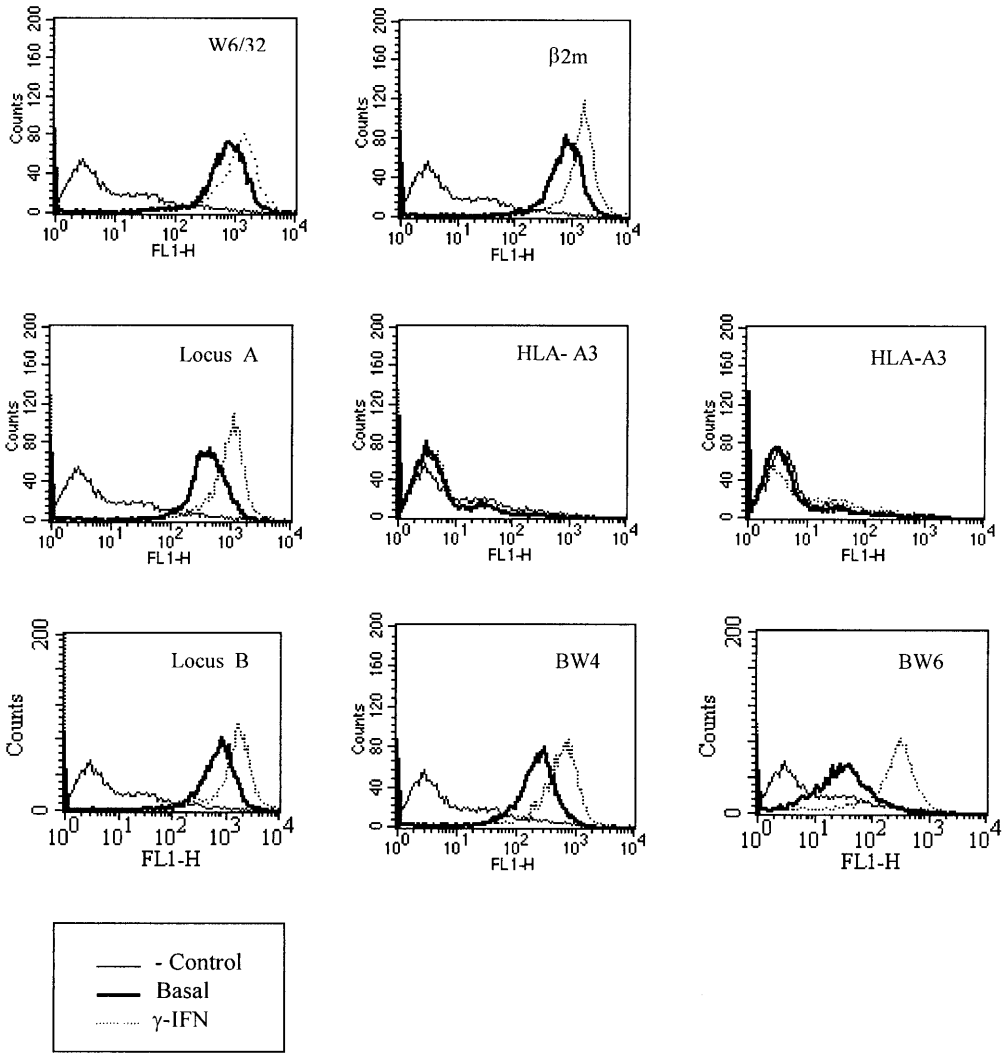

Fig. 1 FACS analysis of HLA class I antigen expression in DU145 cells labeled with monomorphic, locus-specific, and allele-specific mAbs, after incubation in the absence or presence of interferon (IFN)-γ. HLA-A3 surface expression was tested with two allele-specific mAbs

Table 1 Sequences and locations of the primers used for amplifications and sequencing of *HLA*0301* (+ *HLA-A*3*-specific primer, * *HLA-A* locus-specific primers; *S* sense, *AS* antisense)

Primer	Sequence	Location	Orientation
Pra	5′GGGCCCATGCCGAGGGTTT	–385	Sense
A3-AS+	5′GGTCAGTCTGTGACTGGGCCTTCAC	Exon 2	Antisense
A-S*	5′GACAGCGACGCCGCGAGCCA	Exon 2	Sense
A-AS*	5′GGCAGCGACCACAGCTCCAG	Exon 5	Antisense
Int1-S	5′CGAGGGTCGGGCAGGTCTCA	Intron 1	Sense
Int 2-AS	5′CAGCCCCGACCAACCCGGGG	Intron 2	Antisense
Int2-S	5′CCCCGGGTTGGTCGGGGCTG	Intron 2	Sense
Int3-AS	5′CCTCCTTGTGGGAGGCCAGC	Intron 3	Antisense
Ex3-S	5′CCTGGAGAACGGGAAGGAGA	Exon 3	Sense
Ex4-AS	5′CCCGCTGCCAGGTCAGTGTG	Exon4	Antisense
Ex4-S	5′CTGCGGAGATCACACTGACC	Exon 4	Sense

We therefore explored the *HLA-A*0301* gene itself by PCR amplification of genomic DNA with *HLA-A* locus-specific primers (A-S and A-AS), which amplify a 1725-bp product. The purified PCR product was cloned in the PCR 4-TOPO vector using the TOPO TA Cloning Kit for Sequencing (Invitrogen, Groningen, The Netherlands). DNA sequencing of the cloned fragments, using the primer pairs listed in Table 1, showed the insertion of an additional C within a cytosine island located at the beginning of exon 4 (nucleotides 621–627). Figure 2a shows comparative DNA sequencing electropherograms from plasmids containing *HLA-A*0301* and *HLA-A*3303* sequences. The additional nucleotide in the sequence between codons 183 and 185 leads to a premature stop codon at position 196, within exon 4 (Fig. 2b). The same insertion has been described in three different null alleles: *A*0104N* (Laforet et al. 1997), *A*2411N* (Magor et al. 1997), and *B*5111N* (Elsner et al. 2001).

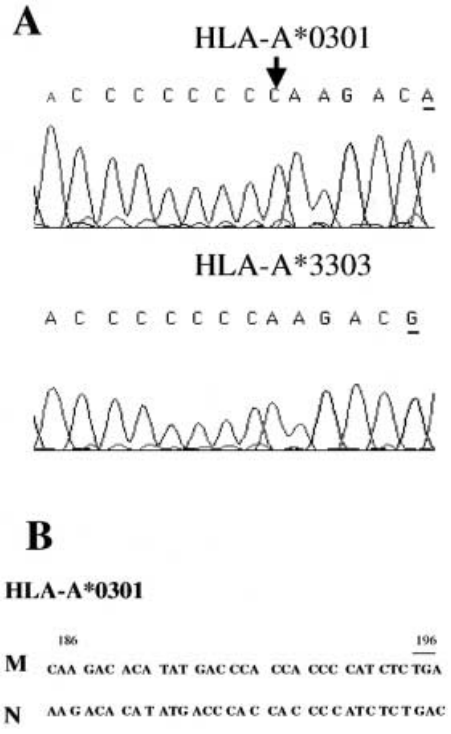

Fig. 2 **a** DNA sequencing electropherograms from plasmids containing *HLA-A3*0301* and *HLA-A*3303* sequences. The *arrow* indicates the cytosine insertion found in the *HLA-A*0301* allele. *Underlined* nucleotides indicate a polymorphic location between *HLA-A*0301* and *HLA-A*3303* alleles. **b** Comparison of the nucleotide sequences at the beginning of exon 4 (codons 186–196) in normal (*N*) and mutated (*M*) *HLA-A*0301*. The premature stop codon generated at position 196 is *overlined*

Lienert and co-workers (1996) described an *HLA-A*0301* null allele named *HLA-A*0303N*. However, in this case, the absence of cellular expression of the HLA-A3 antigen was associated with a six-nucleotide deletion in exon 3 of the *A*0301* gene. In each null allele, the single nucleotide change is the only difference that distinguishes the null allele from the wild type.

The cytosine insertion found in cell line DU145 is the only mutational event detected within the 2.4 kb of genomic DNA sequence determined, and likely accounts for the absence of HLA-A3 antigen surface expression. On the other hand, the available evidence suggests that premature termination codons within mammalian genes reduce the levels of mRNA by a nuclear post-transcriptional mechanism (Carter et al. 1996). Severe reductions in mRNA levels induced by premature termination codons have been described in *HLA* class I genes (Magor et al. 1997; Serrano et al. 2000). This is consistent with the absence of the *HLA-A*0301* transcript when we amplified mRNA from the cell line.

Most null alleles have been characterized in normal cells from healthy individuals. However, we located the inactivating mutation in tumor-derived cells. HLA class I antigens are lost or downregulated in many types of human tumor, and this alteration may permit tumor cells to escape cytotoxic lymphocyte-mediated recognition. Different phenotypes of altered HLA class I antigen expression on tumor cells have been described (Garrido et al. 1997), e.g., total loss or downregulation, haplotype loss, locus downregulation, and allelic loss. Selective allelic loss has been defined in several types of malignant lesion with immunochemical methods that used allele-specific mAbs. The molecular defects underlying this alteration have been characterized in tumor cell lines from colorectal (Browning et al. 1993), cervical (Brady et al. 2000; Koopman et al. 1999), and melanoma (Wang et al. 1999) tumors. The mutations giving rise to specific allelic loss include nucleotide substitutions, and insertion in coding and untranslated regions. Certain genomic regions, particularly sequences with single- or double-base pair repeats, are more prone to undergo mutations such as replication errors caused by strand slipping. For example, a hotspot mutation region has been reported in the highly repetitive sequence CTCTCTCTTTCT located in the leader sequence of the gene for β_2-microglobulin at codon 15–16 of exon 1. Mutations in this region produce a frameshift in the open reading frame, with the appearance of a stop codon at position 42 and a final phenotypic result of total HLA class I loss in a variety of tumors (Perez et al. 1998).

Table 2 Size of PCR products and sequences of the primers used for amplifications of microsatellite markers (*S* sense, *AS* antisense)

Primer	Sequence	Size of PCR product (bp)
BAT26-S	5′TGACTACTTTTGACTTCAGCC	116
BAT26-AS	5′AACCATTCAACATTTTTAACCC	
BAT40-S	5′ATTAACTTCCTACACCACAAC	124
BAT40-AS	5′GTAGAGCAAGACCACCTTG	
BAX-S	5′ATCCAGGATCGAGCAGGGCG	92
BAX-AS	5′ACTCGCTCAGCTTCTTGGTG	
BATRII-S	5′AAGCTCCCCTACCATGACT	114
BATRII-AS	5′TGCACTCATCAGAGCTACAG	

Mismatch repair genes are responsible for the correction of replication errors. In tumor cells with the mutator phenotype, the defective DNA mismatch repair mechanism leads to a high rate of mutation and accumulation of mutations during tumor progression (Ionov et al. 1993). Hence nucleotide repeats are more susceptible to mutation in tumor cells that exhibit the mutator phenotype. The cytosine island located at the beginning of exon 4 of the *HLA-A*0301* gene may be a mutation hotspot in tumor cells with the mutator phenotype. Because tumors with a replication repair defect (RER) display microsatellite instability (change in the length of simple repetitive sequences), we analyzed the DU145 cell line for its RER phenotype using several mononucleotide repeat sequences. Because normal DNA was unavailable for comparison between germline microsatellites and microsatellites from the DU145 cell line, we looked for microsatellite instability in the polyadenine mononucleotide repeats BAT-26 and BAT-40, which have previously been shown to be relatively monomorphic in size distri-

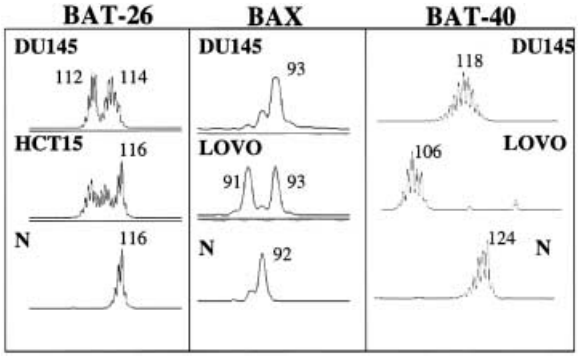

Fig. 3 Comparative microsatellite analysis at *BAT-26*, *BAX*, and *BAT-40* markers. DU145 showed a deletion of two and four nucleotides in *BAT-26* alleles, and a G insertion at the *BAX* locus, in comparison with normal unmatched DNA. DU145 was also unstable at the *BAT-40* locus, showing allele size reduction. HCT15 and LoVo cell lines were used as positive controls of microsatellite instability

bution within the population (Grady et al. 1998; Hoang et al. 1997). In addition, two microsatellite-like mononucleotide repeats were analyzed: a 10-bp polyadenine repeat within the coding region of the transforming growth factor-β (TGF-β) type II receptor (the *BAT-RII* tract), and a tract of eight consecutive deoxyguanosines in the third exon of the *BAX* gene. Mutations in these repeated sequences of both genes have been reported in tumors with the mutator phenotype (Grady et al. 1998; Ionov et al. 2000).

To amplify microsatellite markers, the 5′ site of one of the primers of each primer set was tagged with a fluorescent label (Applied Biosystems). Microsatellite instability was determined using a 377 DNA Sequencer and Genescan 3.1 software (Applied Biosystems). The primer sequences and the size of PCR products are listed in Table 2. *BAT-26* and *BAT-40* were considered to be unstable when they showed size variations exceeding 2 bp in cell line DU145 as compared with larger alleles from unmatched normal DNA. As a positive control, we used HCT-15 and LoVo colorectal cell lines (ATCC), which are known to be unstable for mononucleotide runs (Branch et al. 1995; Hoang et al. 1997). The DU145 cell line was unstable for *BAT-26* and *BAT-40* (Fig. 3), showing reductions in allele size in both markers. As shown in Fig. 3, *BAT-26* amplification from normal unmatched DNA demonstrated the normal-sized PCR fragment (116 bp), whereas the two alleles of *BAT-26* in cell line DU145 showed deletions of two and four bases (114 and 112 bp). Moreover, we detected a single nucleotide insertion in the G_8 tract of the *BAX* gene (Fig. 3), whereas the size of the *BAT-RII* tract was normal (data not shown).

The mutational event that gives rise to loss of expression of *HLA-A*0301* in cell line DU145 may be interpreted in two ways: (1) the insertion of an extra cytosine generates a novel *HLA-A3* null allele or (2) the inactivating mutation arises from tumor genomic instability and is selected during tumor progression because it represents an immunological advantage. According to the first possibility, the mutational event should be present in germline DNA. Unfortunately, normal matching DNA was unavailable to test the two hypotheses.

In summary, we described a new structural mutation in an *HLA* class I gene that causes a lack of transcription and surface antigen expression. These results reveal that is important to compare phenotype and genotype in order to understand the molecular basis of variations in HLA expression.

Acknowledgements We thank Carmen Amezcua and M. José Guadix for expert technical assistance, and K. Shashok for checking the English. This work was supported by the Fondo de Investigaciones Sanitarias, Servicio Andaluz de Salud, Spain (P.J., F.R.-C., and F. G.) and the Fundación para la Investigación en Urología.

References

Brady CS, Bartholomew JS, Burt DJ, Duggan-Keen M, Glenville S, Telford N, Little AM, Davidson JA, Jiménez P, Ruiz-Cabello F, Garrido F, Stern PL (2000) Multiple mechanisms underlie HLA dysregulation in cervical cancer. Tissue Antigens 55:401–411

Branch P, Bicknell DC, Rowan A, Bodmer WF, Karran P (1995) Immune surveillance in colorectal carcinoma. Nat Genet 9:231–232

Browning MJ, Krausa P, Rowan A, Hill AB, Bicknell DC, Bodmer JG, Bodmer WF (1993) Loss of human leukocyte antigen expression on colorectal tumor cell lines: implications for anti-tumor immunity and immunotherapy. J Immunother 14:163–168

Carter MS, Li S, Wilkinson MF (1996) A splicing-dependent regulatory mechanism that detects translation signals. EMBO J 15:5965–5975

Elsner HA, Drabek J, Rebmann V, Ambruzova Z, Grosse-Wilde H, Blasczyk R (2001) Non-expression of HLAB*5111N is caused by an insertion into the cytosine island at exon 4 creating a frameshift stop codon. Tissue Antigens 57:369–372

Garrido F, Ruiz-Cabello F, Cabrera T, Perez-Villar JJ, López-Botet M, Duggan-Keen M, Stern PL (1997) Implications for immunosurveillance of altered HLA class I phenotypes in human tumours. Immunol Today 18:89–95

Grady WM, Rajput A, Myeroff L, Liu DF, Kwon K, Willis J, Markowitz S (1998) Mutation of the type II transforming growth factor-β receptor is coincident with the transformation of human colon adenomas to malignant carcinomas. Cancer Res 58:3101–3104

Hoang JM, Cottu PH, Thuille B, Salmon RJ, Thomas G, Hamelin R (1997) BAT-26, an indicator of the replication error phenotype in colorectal cancers and cell lines. Cancer Res 57:300–303

Ionov P, Peinado MA, Malkhosyan S, Shibata D, Perucho M (1993) Ubiquitious somatic mutations in simple repeated sequences reveal a new mechanism for colonic carcinogenesis. Nature 363:558–561

Ionov Y, Yamamoto H, Krajewski S, Reed JC, Perucho M (2000) Mutational inactivation of the proapoptotic gene BAX confers selective advantage during tumor clonal evolution. Proc Natl Acad Sci USA 97:10872–10877

Koopman LA, Silk AR van der, Giphart MJ, Fleuren GJ (1999) Human leukocyte antigen class I gene mutations in cervical cancer. J Natl Cancer Inst 91:1669–1677

Laforet M, Froelich N, Parissiadis A, Pfeiffer B, Schell A, Faller B, Woehl-Jaegle ML, Cazenave JP, Tongio MM (1997) A nucleotide insertion in exon 4 is responsible for the absence of expression of an HLA-A*01 allele. Tissue Antigens 50:347–350

Lardy NM, Bakas RM, Horst AR van der, Twuyver E van, Bontrop RE, Waal LP de (1992) Cis-acting regulatory elements abrogate allele-specific HLA class I gene expression in healthy individuals. J Immunol 148:2572–2577

Lienert K, Russ G, Lester S, Bennett G, Gao X, McCluskey J (1996) Stable inheritance of an "blank" phenotype associated with a structural mutation in the HLA-A*0301 gene. Tissue Antigens 48:187–91

Magor KE, Taylor EJ, Shen SY, Martinez-Naves E, Valiante NM, Spencer Wells R, Gumperz JE, Adams EJ, Little AM, Williams F, Middleton D, Gao X, McCluskey J, Parham P, Weidenbach KL (1997) Natural inactivation of a common HLA allele A*2402 has occurred on at least three separate occasions. J Immunol 158:5242–5250

Parham P (1997) Filling in the blanks. Tissue Antigens 50:318–321

Pérez B, Benitez R, Fernández MA, Oliva MR, Soto JL, Serrano S, López-Nevot MA, Garrido F (1998). A new β2 microglobulin mutation found in a melanoma tumor cell line. Tissue Antigens 53:569–572

Serrano A, Brady CS, Jiménez P, Duggan-Keen MF, Méndez R, Stern P, Garrido F, Ruiz-Cabello F (2000) A mutation determining the loss of HLA-A2 antigen expression in a cervical carcinoma reveals novel splicing of human MHC class I classical transcripts in both tumoral and normal cells. Immunogenetics 51:1047–1052

Wang Z, Marincola FM, Rivoltini L, Parmiani G, Ferrone S (1999) Selective histocompatibility leukocyte antigen (HLA)-A2 loss caused by aberrant pre-mRNA splicing in 624MEL28 melanoma cells. J Exp Med 190:205–215