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Tissue typing the *HLA-A* locus from genomic DNA by sequence-specific PCR: Comparison of *HLA* genotype and surface expression on colorectal tumor cell lines

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ABSTRACT **A system devised for tissue typing the *HLA-A* locus by PCR from genomic DNA has been used to investigate abnormalities of HLA expression in a panel of 30 colorectal tumor cell lines, by comparing the *HLA-A* locus genotype with surface expression of HLA. Three cell lines showed complete lack of HLA expression associated with failure to express β_2-microglobulin. In two other cell lines, loss of expression of *HLA-A2* was observed, in spite of the presence of the gene in genomic DNA. Eleven cell lines gave a single *HLA-A* locus specificity on PCR typing. In one of these cell lines we have demonstrated the loss of an *HLA-A* locus gene in the tumor cell by comparison with DNA from a lymphoblastoid B-cell line derived from the same patient. These data indicate that at least three independent mechanisms were involved in the loss of HLA expression on the colorectal tumor cell lines.**

HLA class I molecules are transmembrane glycoproteins consisting of two polypeptide chains. The class I heavy chains are encoded by three principal genes (*HLA-A*, *-B*, and *-C*) within the HLA region of chromosome 6. The products of these genes are associated at the cell surface with a nonpolymorphic light chain, β_2-microglobulin (β_2m), and a cell-derived peptide to form a stable complex (1). Surface expression of this complex controls the recognition of a cell by antigen-specific cytotoxic T lymphocytes (2, 3) and has been implicated in the rejection of intracellular parasites (4, 5), tumors (6, 7), and tissue allografts (8). Loss of expression of HLA molecules has been described on a wide variety of tumor types (9–13) and has been argued as presenting a mechanism whereby a tumor cell can escape from immune surveillance by cytotoxic T lymphocytes (14, 15). To better understand the control of HLA expression on tumor cells, we have established a DNA-based method for tissue typing the *HLA-A* locus by a one-step PCR from genomic DNA. *HLA-A* locus-specific amplification of target DNA is achieved by the use of combinations of sequence-specific oligonucleotide primers, to yield PCR products of defined size. The specificity of priming the PCR is determined by the amplification refractory mutation system, in which nonspecific reactions are inhibited by nucleotide mismatch at the 3′ residue of the primer (16). A panel of oligonucleotide primers has been assembled that allows the identification of *HLA-A* locus specificities *HLA-A1*, *-A2*, *-A3*, *-A9*, *-A11*, *-A25*, *-A26*, *-A28*, *-A29*, *-A30*, *-A31*, *-A32*, and *A33*, and the group-specific typings *-A10* and *-A19* (except *A30*) (17). We have used this system to define the *HLA-A* locus genotype of a panel of 30 cell lines derived from colorectal adenocarcinomas and to compare this with the surface expression of HLA class I, in general, and of HLA-A2, in particular.

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MATERIALS AND METHODS

Cell Lines and Extraction of Genomic DNA. The majority of the colorectal tumor cell lines have been described (see Table 3). Colorectal cell lines C10, C32, C70, C75, C80, and C84 were established in this laboratory from surgically resected colorectal adenocarcinomas as described (18). Extraction of genomic DNA from cell pellets was done manually (19) or by using an Applied Biosystems 340A nucleic acid extractor and standard methods.

***HLA-A* Locus-Specific PCR.** *HLA-A* locus-specific PCR was done by using a panel of nine coding-strand and seven noncoding-strand primers. HLA-specific primer sequences were derived from HLA class I nucleotide sequences (20). The nucleotide sequences of the primers are shown in Table 1. *HLA-A* locus-specific amplification was achieved by using combinations of primers as listed in Table 2. Amplification of genomic DNA yielded *HLA-A* locus-specific PCR products ranging in size from 397 to 631 bp (Table 2). Each reaction included positive control primers, specific for the gene encoding β_2m (Table 1), which amplified a smaller (330 bp), and so readily distinguishable, product. A negative control reaction, containing distilled water in place of DNA, was run with each typing set.

Each of the PCR reactions was done in 50-μl vol, containing 17 mM ammonium sulfate, 67 mM Tris·HCl (pH 8), 6.7 μM EDTA, 0.017% bovine serum albumin, 200 μM of each dNTP, 2 mM magnesium chloride, 0.2 μM of each of the two control primers and two relevant sequence-specific primers, and 1 μg of target DNA. The reaction mix was heated to 95°C for 5 min for DNA denaturation and cooled to room temperature; the condensation was then spun down before addition of 1 unit of Amplitaq (Perkin–Elmer/Cetus) and overlaying with mineral oil. The amplifications were done in a Hybaid (Teddington, U.K.) thermal cycler over 30 cycles, each of 95°C (1 min), 55°C (1 min), and 72°C (2 min). After the PCR, 10 μl of each reaction was run out on a 2% agarose gel. The *HLA-A* locus tissue type was determined by the presence or absence of the appropriate-sized bands on the gel for each specific reaction. The specificity of the primer combinations for each of the *HLA-A* locus-specific PCR reactions was validated against DNA from a panel of lymphoblastoid B-cell lines of known tissue type (data not shown).

HLA-Specific ELISA and Flow Cytometry. Monoclonal antibodies (mAbs) (and their specificities) used in the study were W6/32 (HLA class I—β_2m complex; ref. 21), L368 (β_2m; ref. 22) and BB7.2 (HLA-A2, -A69; ref. 9). The galactosidase/antigalactosidase ELISA method has been described (23). Staining of the cells was expressed in arbitrary ELISA units on a scale from 0 to 4000. An ELISA reading of <300 correlated with negative staining of the cell on flow

Abbreviations: β_2m, β_2-microglobulin; mAb, monoclonal antibody.
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Table 1. Oligonucleotide primer sequences

Primer	Sequence (5′–3′)	T_m
Coding		
AL#2	ACA GAC TGA CCG AGC GAA	56
AL#3	GAC GGG GAG ACA CGG AAA	58
AL#4	GAG TAT TGG GAC CGG AAC	56
AL#5	CAC TCC ATG AGG TAT TTC TC	58
AL#6	CGG AAT GTG AAG GCC CAG	58
AL#7	GCG ACG CCG CGA GCC A	58
AL#10	GAT AGA GCA GGA GAG GCC T	60
AL#11	CAC AGA CTG ACC GAG AGA G	60
AL#15	CCT GCG GAT CGC GCT CC	60
Noncoding		
AL#A	CTC CAG GTA GAC TCT CCG	58
AL#C	TGT AAT CCT TGC CGT CGT AA	58
AL#D	CAC TCC ACG CAC GTG CCA	60
AL#F	GCG CAG GTC CTC GTT CAA	58
AL#H	CAA GAG CGC AGG TCC TCT	58
AL#I	TCT CTG CTG CTC CGC CG	58
AL#K	TCT CAA CTG CTC CGC CCA	58
Internal control (β_2m)		
Coding	CGA TAT TCC TCA GGT ACT	52
Noncoding	CAA CTT TCA GCA GCT TAC	52

cytometric analysis. Flow cytometry was done on a Becton Dickinson FACScan, after staining of the cells with mAbs and labeling with fluorescein isothiocyanate-conjugated F(ab′)$_2$ fragment of rabbit anti-mouse IgG (Dako, High Wycombe, U.K.).

RESULTS

***HLA-A* Locus-Specific PCR Typing of Colorectal Tumor Cell Lines.** For practical purposes, genomic DNA was screened by using a low-resolution panel of nine reactions, corresponding to *HLA-A* locus specificities *HLA-A1, -A2, -A3, -A9, -A10, -A11, -A28, -A19* (except *A30*), and *-A30*. Subtyping of the group-specific reactions *HLA-A10* (*-A25* and *-A26*) and *-A19* (*-A29, -A31, -A32, -A33*) was done as a separate step, as required.

This method was used to identify the *HLA-A* locus genotype of a panel of 30 colorectal tumor cell lines. This panel included three pairs of cells (COLO 201 and COLO 206, DLD-1 and HCT 15, and HT29 and WIDR) that were derived from the same individuals. Representative typings of three colorectal cell lines are shown in Fig. 1, and a list of the *HLA-A* locus specificities of all the cell lines is given in Table 3. Eleven of the colorectal cell lines from 27 individuals showed a single *HLA-A* locus specificity. This incidence is higher than predicted for homozygosity of *HLA-A* locus alleles in the general population. A comparison with the number of homozygotes seen in a random population (13 of 91) gives a χ^2 for the significance of the difference of 7.4. The difference between the expected frequency of homozygosity among the tumor cell lines and that observed gives an estimated frequency of allele loss of 30%. Patient-matched Epstein–Barr virus-transformed lymphoblastoid B-cell lines were available for three of the colorectal cell lines. A comparison of the PCR typings of the matched colorectal tumors and B-cell lines is shown in Fig. 2. For colorectal tumor cell lines HCA-7 and HCA-46, the *HLA-A* locus genotype corresponded with that of the relevant matched B-cell line EVA-1224 and IRM-1223, respectively. The tumor cell line LS 411, however, consistently typed as HLA-A2 only, whereas the corresponding B-cell line RN typed as HLA-A2, -A11. The cell line HCA-7, which typed as *HLA-A1, -A2* by PCR, has been described as failing selectively to express

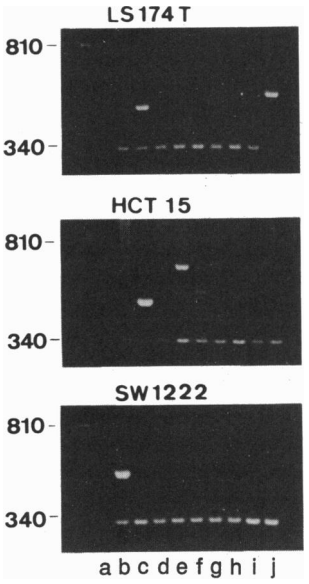

FIG. 1. *HLA-A* locus PCR typings of colorectal tumor cell lines LS 174T, HCT 15, and SW 1222. Individual photographs represent negative control (lane a) and *HLA-A* locus-specific reactions for *HLA-A1, -A2, -A3, -A9, -A10, -A11, -A28, -A19* (except *-A30*) and *-A30* (lanes b–j, respectively). Numbers at left indicate bp.

Table 2. HLA-A locus-specific primer combinations

HLA-A locus specificity	Coding primer	Noncoding primer	Specificity of coding primer	Specificity of noncoding primer	Band size, bp
A1	AL#2	AL#A	**A1** A26 A29	**A1**	510
A2	AL#3	AL#H	**A2**	A9 **A2** A28	457
A3	AL#6	AL#D	A11 **A3** A28 A29 A30	**A3**	547
A9	AL#5	AL#H	**A9** HLA-G	**A9** A2 A28	631
A10	AL#7	AL#C	HLA-A except A202 A205	**A10**	497
A25	AL#11	AL#C	**A25** A32 multi HLA-B	**A25** A26	398
A26	AL#2	AL#C	A1 **A26** A29	A25 **A26**	397
A11	AL#6	AL#I	**A11** A3 A28 A29 A30	A1 **A11**	518
A28	AL#6	AL#H	A11 A3 **A28** A29 A30	A9 A2 **A28**	445
*A19**	AL#7	AL#F	HLA-A except A202 A205	**A29 A31 A32 A33**	520
A29	AL#2	AL#F	A1 A26 **A29**	**A29** A31 A32 A33	420
A30	AL#6	AL#K	A11 A3 A28 A29 **A30**	**A30**	518
A31	AL#10	AL#F	A30 **A31**	A29 **A31** A32 A33	480
A32	AL#15	AL#F	A9 A25 **A32** multi HLA-B	A29 A31 **A32** A33	402
A33	AL#4	AL#F	A28 A10 **A33** HLA-H	A29 A31 A32 **A33**	461

Boldface letters indicate matching specificities of coding and noncoding primers.
*Recognizes all *HLA-A19* specificities except *-A30*.

Table 3. Comparison of *HLA-A* locus genotype and surface expression of HLA on colorectal tumor cell lines

Cell line	*HLA-A* locus PCR typing	ELISA,† units W6/32	L368	BB7.2	Ref.
C10	*A2*, –	769	3117	316	
C32	*A9*, *A10(26)*	958	3571	36	
C70	*A28*, *A30*	1710	4000	73	
C75	*A1*, *A11*	1813	4000	59	
C80	*A3*, –	1588	4000	95	
C84	*A2*, *A3*	ND	ND	ND	
CC07	*A2*, –	3700	4000	1685	24
CC20	*A1*, –	2714	4000	121	25
COLO 201*	*A1*, *A2*	4000	4000	1142	26
COLO 206*	*A1*, *A2*	3521	4000	537	26
DLD-1*	*A2*, *A9*	75	92	111	27
HCA 7	*A1*, *A2*	4000	4000	2115	28
HCA 46	*A1*, *A19(32)*	2508	4000	90	28
HCT 15*	*A2*, *A9*	0	0	31	27
HT 29*	*A1*, *A9*	3389	4000	24	29
JW	*A2*, –	1506	4000	1006	30
LIM 1863	*A3*, *A11*	1134	3004	95	31
LoVo	*A1*, *A19(32)*	ND	ND‡	ND	9
LS 174T	*A2*, *A30*	670	4000	76	32
LS 411	*A2*, –	1155	4000	88	33
LS 1034	*A2*, *A10(26)*	4000	4000	964	33
SW 48	*A9*, –	141	184	174	34
SW 403	*A2*, *A3*	2926	4000	369	34
SW 837	–, *A19(32)*	4000	4000	176	34
SW 1222	*A1*, –	1312	4000	96	34
SW 1417	*A3*, –	4000	4000	105	34
T84	*A2*, *A9*	3813	4000	735	35
Vaco 4A	*A1*, *A3*	986	2564	183	18
Vaco 5	*A1*, –	1601	4000	144	18
WiDr*	*A1*, *A9*	2184	4000	167	36

ND, not done.
*Cell lines COLO 201 and COLO 206, HT29 and WiDr, and DLD-1 and HCT 15, respectively, are paired cell lines derived from the same individuals.
†ELISA units are expressed on a scale from 0 to 4000. An ELISA reading of <300 correlated with negative staining on flow cytometry.
‡These data have been reported as β_2m deficient (9, 37).

HLA-A1 (15). The PCR confirmed the presence of the *HLA-A1* gene in this cell line, indicating that the loss of expression of HLA-A1 is by a mechanism other than gene loss.

Comparison of *HLA-A* Locus Genotype and Surface Expression of HLA. HLA expression on the colorectal tumor cell lines was determined by ELISA using mAbs W6/32, L368, and BB7.2 and was confirmed on selected cell lines by flow cytometry. Details of the expression of HLA class I, β_2m and HLA-A2 are given in Table 3. Three cell lines (DLD-1, HCT 15, and SW48) showed lack of expression of HLA class I, associated with low or undetectable expression of β_2m. Absence of staining with mAb W6/32 was confirmed by flow cytometry (data not shown). The data suggest that the loss of HLA expression in these cell lines was due to failure to express β_2m. This result is supported by the report that the cell line HCT 15 has mutations in both β_2m genes, resulting in its failure to express an intact β_2m gene product (38). The cell lines HCT 15 and DLD-1 were derived from the same patient and share the phenotype of lack of HLA expression and absent staining for β_2m. In two other cell lines (LS 174T and LS 411), mAb W6/32 staining was positive by ELISA, but mAb BB7.2 (HLA-A2) staining was absent in spite of positive reactions for the *HLA-A2* gene by PCR (Figs. 1 and 2). Flow cytometric analysis confirmed the lack of surface expression of HLA-A2 (data not shown). This finding suggests a selective loss of expression of the *HLA-A2* gene products in these cell lines.

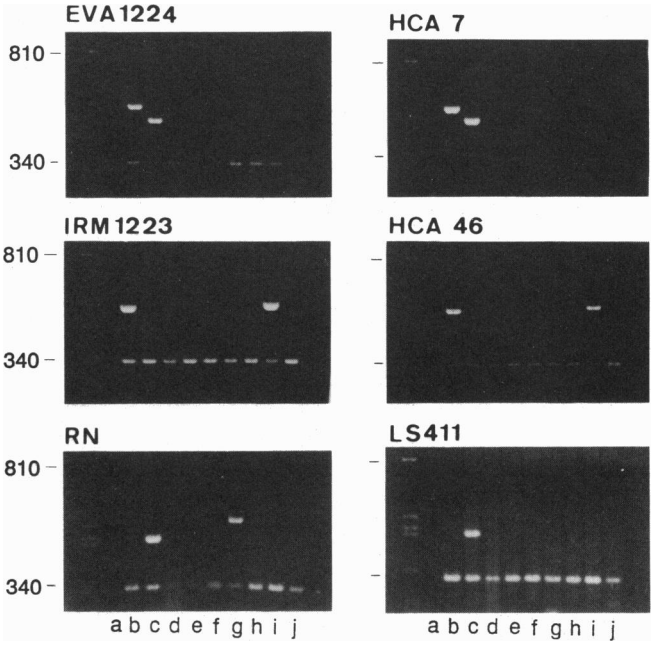

FIG. 2. *HLA-A* locus PCR typings of colorectal tumor cell lines HCA 7, HCA 46, and LS 411 and patient-matched lymphoblastoid B-cell lines EVA 1224, IRM 1223, and RN, respectively. Individual photographs represent negative control (lane a) and *HLA-A* locus-specific reactions for *HLA-A1*, *-A2*, *-A3*, *-A9*, *-A10*, *-A11*, *-A28*, *-A19* (except *-A30*), and *-A30* (lanes b–j, respectively). Numbers at left indicate bp.

DISCUSSION

We have devised a method for typing the *HLA-A* locus by PCR from genomic DNA and have used it to compare the *HLA* genotype with HLA expression on a panel of colorectal tumor cell lines. DNA-based methods have not previously been available for tissue typing an HLA class I gene locus. The use of combinations of HLA class I sequence-specific primers, designed along the principle of the amplification refractory mutation system (16), permits the *HLA-A* locus-specific amplification of genomic DNA by PCR (17). The method is capable of distinguishing *HLA* alleles based on a single nucleotide difference. This degree of sensitivity may have important implications for tissue typing for transplantation, as serological typing for class I fails to identify all allelic variants of HLA specificities.

We have used the technique to identify abnormalities of HLA expression in a panel of colorectal tumor cell lines. Loss of HLA expression has been described on a wide range of tumor types (9–13), but analysis of the underlying mechanism has been limited. The high incidence of single *HLA-A* locus specificities observed in our study suggests that gene loss may be a common mechanism for loss of HLA expression on tumor cells. The loss of the *HLA-A11* gene in cell line LS 411, as compared with its B-cell partner cell line RN, supports this theory. Although we cannot rule out that mutations at a site of PCR primer binding may underlie the phenomenon, the most likely explanation is the loss of *HLA* genes secondary to chromosome loss (nondisjunction) or somatic recombination (39). A similar phenomenon appears to have occurred in the cell line HeLa, which has been typed as HLA-A3, -A28 by family studies (40) and somatic cell hybridization. *HLA-A* locus-specific PCR analysis of this cell line and its derivative D98, consistently gave the *HLA-A*

locus genotype as *HLA-A28* only, suggesting the loss of the *HLA-A3* gene. The same phenomenon probably also explains some of the combined *-A* and *-B* locus losses described (15).

The selective loss of expression of individual HLA molecules on tumor cells has been described (13, 41). In this study we have identified two cell lines (LS 174T and LS 411), in which loss of expression of HLA-A2 has occurred. LS 411 has, therefore, two genetic changes with respect to *HLA-A* locus expression because it has also lost *-A11*. In addition, loss of expression of HLA-A1 on the cell line HCA-7 has been described (15). Preliminary investigation by PCR has shown the presence of mRNA for HLA-A1 and HLA-A2 in cell lines HCA-7 and LS 174T, respectively, indicating that the failure of expression is by a mechanism other than transcriptional regulation of the gene.

The complete loss of HLA expression, resulting from failure to express β_2m, was identified in colorectal tumor cell lines derived from two individuals and has been described (9) on one other cell line (LoVo) in this study. Our *HLA-A*-locus PCR-typing method has allowed us to define the *HLA* genotype of these cells, in spite of the absence of expression of any HLA molecules. We have also used the technique to confirm the *HLA-A* locus genotype (*HLA-A1, -A10*) of the Burkitt lymphoma-derived cell line Daudi, the prototypic example of β_2m loss in a tumor cell line (37).

It has been argued that loss of HLA expression on a tumor cell represents a means of evasion of the immune response by the tumor, with consequent immunoselection of the mutant cell (14, 15). Molecular analysis of the mechanisms of HLA loss on these tumor cell lines may give insights into the extent to which this might occur. In addition, as prospects for T-cell-based immunotherapy against cancer improve, the failure of a tumor to express particular HLA molecule(s) may prove an important barrier to the successful eradication of tumor cells by immunological means (42).

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