

## The *LMP7-K* Allele of the Immunoproteasome Exhibits Reduced Transcript Stability and Predicts High Risk of Colon Cancer

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### Abstract

Destruction of cancer cells by cytotoxic T lymphocytes depends on immunogenic tumor peptides generated by proteasomes and presented by human leukocyte antigen (HLA) molecules. Functional differences arising from alleles of immunoproteasome subunits have not been recognized so far. We analyzed the genetic polymorphism of the immunoproteasome subunits LMP2 and LMP7 and of the transporters associated with antigen processing (TAP1 and TAP2) in two independently collected panels of colorectal carcinoma patients ( $N_1 = 112$ ,  $N_2 = 62$ ; controls,  $N = 165$ ). High risk of colon cancer was associated with the *LMP7-K/Q* genotype (OR = 8.10,  $P = 1.10 \times 10^{-11}$ ) and low risk with the *LMP7-Q/Q* genotype (OR = 0.10,  $P = 5.97 \times 10^{-13}$ ). The basis for these distinct associations of LMP7 genotypes was functionally assessed by IFN- $\gamma$  stimulation of colon carcinoma cell lines ( $N = 10$ ), followed by analyses of mRNA expression of *HLA* class I, *TAP1*, *TAP2*, and *LMP7*, with real-time PCR. Whereas induction of *HLA-B*, *TAP1*, and *TAP2* was comparable in all cell lines, transcript amounts of *LMP7-Q* increased 10-fold, but of *LMP7-K* only 3.8-fold. This correlated with a reduced transcript stability of *LMP7-K* ( $t_{1/2} \approx 7$  minutes) compared with *LMP7-Q* ( $t_{1/2} \approx 33$  minutes). In addition, *LMP7-Q/Q* colon carcinoma cells increased (the peptide based) HLA class I surface expression significantly after IFN- $\gamma$  stimulation, whereas *LMP7-Q/K* and *LMP7-K/K* carcinoma cells showed minimal (<20%) changes. These results suggest that the presence of *LMP7-K* can reduce the formation of immunoproteasomes and thus peptide processing, followed by reduced peptide-HLA presentation, a crucial factor in the immune response against cancer. *Cancer Res*; 71(23); 7145–54. ©2011 AACR.

### Introduction

Genetic and environmental factors influence type and growth of malignant tumors. Numerous studies have found associations between particular human leukocyte antigen (HLA) alleles and an increased risk to develop cancer, and the loss of HLA expression by tumors has been associated with poor prognosis in many tumors (1, 2). The initiation of successful antitumor immune responses requires presentation of immunogenic tumor peptides by HLA classes I and II mole-

cules (3). Efficient presentation of peptide-HLA complexes (pMHC) on the cell surface depends on the production and processing of the peptide. Proteasomes and immunoproteasomes process intracellular proteins into peptides, whereas selection and transport is carried out by the heterodimeric transporter associated with antigen processing (TAP; ref. 4). IFN- $\gamma$  stimulation alters proteolytic activity and peptide generation of the proteasome through the incorporation of LMP2 (PSMB9) and LMP7 (PSMB8) subunits into newly assembling immunoproteasomes (5). The LMP-containing immunoproteasomes generate more and better fitting peptides than constitutive proteasomes (6).

The HLA informatics group and the Human Genome Organization (HUGO) Gene nomenclature committee established the actual name for LMP2 as PSMB9 [proteasome (prosome, macropain) subunit,  $\beta$  type, 9 (large multifunctional peptidase 2)] and for LMP7 as PSMB8 [proteasome (prosome, macropain) subunit,  $\beta$  type, 8 (large multifunctional peptidase 7; refs. 7, 8)].

Until now studies on dysregulation of antigen processing in cancer cells have focused on the loss or downregulation of TAP or LMP, which can lead to low expression or absence of the pMHC and thereby prevent activation of cytotoxic T lymphocytes (9, 10). Genetic polymorphisms have been identified in LMP7 and LMP2 as well as in the transporter proteins TAP1 and TAP2 (11–13). We hypothesized that the function of the

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antigen processing and transport pathway might be influenced by structural differences encoded by *TAP* or *LMP* alleles and that particular alleles might be associated with increased tumor risk (9). To this end, we first assessed frequencies of *TAP* and *LMP* alleles in patients with colorectal carcinomas compared with healthy controls. Then, we analyzed cancer cells carrying cancer-associated alleles functionally. We compared inducibility of disease-associated *LMP7* alleles and non-disease-associated *LMP7* alleles following IFN- $\gamma$  stimulation by quantifying *LMP7* RNA transcripts and analyzing their stability. Finally, we also measured surface expression of HLA molecules in those cell lines.

## Materials and Methods

### Carcinoma samples, cell lines, and subjects

Historical samples from the large intestine of 112 unrelated Caucasian patients were diagnosed histologically as carcinomas and resected in the following locations: 4 in the cecum, 48 in the colon, 19 in the sigma, 18 in the recto-sigmoidal part, and 23 in the rectum. Twenty-five samples of the colon and 11 of the sigma were snap frozen, all other samples were preserved in paraffin. A second independently collected panel from the Munich area consisted of 62 snap-frozen carcinoma samples, 9 from the cecum, 24 from the colon, 10 from the sigma, 2 from the rectosigmoidal part, and 17 from the rectum. Peripheral blood mononuclear cell (PBMC) from 165 randomly selected unrelated Caucasian individuals from the Munich area with previously defined HLA class I polymorphisms (14) were used as controls. For the control persons, the male/female ratio was 0.64 and age  $33.6 \pm 9.4$  years; for the carcinoma patients, male/female ratio was 0.44 and age  $69.6 \pm 10.3$  years. The control persons were free of disease at the time point of blood collection. No follow-up of the cohorts were available.

Cell lines WT100BIS and KAS116 as well as Caski and HeLa were obtained from the American Type Culture Collection and served as positive controls for the LMP-ARMS-PCRs and human papilloma virus (HPV)-specific PCRs, respectively. Colorectal carcinoma lines Colo320DM, SK-Co-1, SW403, Lovo, LS174T, Colo205, HT29, CX1, WiDr, SW948, and Caco2 were obtained from the European Type Tissue Culture Collection. The colon carcinoma cell line WEB2 was established in the Institute of Immunology, Munich.

### Cell culture

Colorectal cancer cell lines were cultured at 37°C and 5% CO<sub>2</sub> in RPMI 1640 (Boehringer Ingelheim), supplemented with 10% fetal calf serum. For HLA expression, cancer cell lines were treated for 16 or 20 hours with 200 U/mL recombinant human IFN- $\gamma$ -1b (Imukin; Boehringer Ingelheim).

### DNA preparation

PBMCs of control individuals were isolated by ficoll density gradient centrifugation (PAA Laboratories). Genomic DNA of  $1 \times 10^6$  PBMCs or tumor cells was recovered with the Proteinase K method (15). DNA of shock-frozen tumor samples was isolated using the Promega Wizard DNA Purification Kit

(Promega) according to manufacturer's instructions. Paraffin-embedded tumor samples were deparaffinized with xylene according to the protocol of PeqLab (PeqLab), DNA preparation was carried out with the Promega Wizard DNA Purification Kit (Promega).

### LMP-ARMS-PCR

We used the oligonucleotides LMP2-1, LMP2-3, and LMP2-4 as described by Hopkins and colleagues, and Deng and colleagues followed the described procedure to distinguish *LMP2-R* and *LMP2-H* alleles, which have arginine and histidine, respectively, at amino acid (aa) position 60 (accession number X66401; refs. 11, 16). For a better separation of PCR products, the LMP2-2 oligonucleotide was substituted by the following one: 5'-gcc Agc AAg Agc cgA AAc AAg-3'. For *LMP7*, the previously described oligonucleotide and identification procedures were used, which enabled us to distinguish between *LMP7-Q* (glutamine) and *LMP7-K* (lysine) differing in aa position 49 of the prosequence (accession number Z14982; ref. 12). The allelic controls for both ARMS-PCR protocols were carried out by DNA of the cell lines WT100BIS (*LMP2-H/H* or *LMP7-Q/Q*) and KAS116 (*LMP2-R/R* or *LMP7-K/K*) with defined genotypes (16).

### Fluorescence-activated cell-sorting analysis of HLA expression of IFN-stimulated cancer cells

Cells were harvested after 20 hours of incubation with or without IFN and stained according to the standard protocol using fluorescein isothiocyanate-labeled mouse anti-HLA-A, B, C (W6/32; Pharmingen). Samples were analyzed on a Becton-Dickinson FACScan analyzer running CellQuest software (Becton-Dickinson). Each analysis of HLA expression was conducted with 10,000 cells. Relative mean fluorescence intensity (MFI) was used to compare induction rates of HLA class I expression by IFN and calculated as follows: relative MFI (%) = (treated MFI-untreated MFI)/untreated MFI  $\times$  100%.

### Quantitation of LMP7 RNA by reverse transcriptase real-time PCR

Cells were harvested after 16 hours (or appropriate time for mRNA-stability experiment) of incubation with or without IFN, centrifuged and immediately shock frozen in liquid nitrogen. Complete RNA was isolated using the QIAshredder and RNeasy Kit including DNase digestion according to manufacturer's instruction (QIAGEN). For reverse transcription of complete RNA, the RevertAid First Strand cDNA synthesis Kit including random hexamers was chosen, following manufacturer's instruction (Fermentas).

All materials for the reverse transcriptase real time PCR were obtained from Applied Biosystems, using the TaqMan Universal PCR Master Mix, and the following predesigned gene expression assays: *LMP7* exon 1 (Hs00188149\_m1), *LMP7* exon 3 (Hs00544758\_m1), *G6PDH* endogenous control (Hs00166169\_m1), *18S rRNA* endogenous control, *TAP1* (Hs00184465\_m1), *TAP2* (Hs00241060\_m1), and *HLA-B* (Hs00741005\_g1). To detect the *LMP7* allelic variation in exon 2, the oligonucleotides LMP7-E2-fw (5'-TCG GAC CCA GGA

CAC TAC AGT T-3'), LMP7-K-rev (5'-GAC TGG AAG AAT TCT GTG GGA TT-3'), and LMP7-Q-rev (5'-GAC TGG AAG AAT TCT GTG GGA TG-3'), respectively, were used for amplification. LMP7-E2a (5'-ATG CGA TCT CCA GAG CT-3') served as probe. All probes were labeled with 6FAM dye-MGB. Real time-PCR was conducted according to manufacturer's instructions. For each RNA sample, a difference in threshold cycle values ( $\Delta C_t$ ) was calculated: taking the mean  $C_t$  of triplicate tubes and subtracting the mean  $C_t$  of the triplicate tubes for the reference RNA (G6PD or 18 sRNA) measured on an aliquot from the same RT reaction. The  $\Delta C_t$  of the unstimulated sample was then subtracted from the  $\Delta C_t$  for the stimulated sample to generate a  $\Delta\Delta C_t$ . For the used  $\Delta\Delta C_t$  calculation to be valid, the efficiency of the target amplification and the efficiency of the reference amplification must be approximately equal. This was verified in validation experiments (conducted according to manufacturer's instructions), confirming that the absolute value of the slope of log input amount versus  $\Delta C_t$  was below 0.1 (data not shown).

#### Determination of mRNA stability by actinomycin D treatment

Cell culture cells were stimulated with IFN- $\gamma$ -1b as described. Following the addition of 50  $\mu$ g/mL actinomycin D, cells were kept under stimulation condition and samples were taken after 0 minute, 30 minutes, 1 hour, and 4 hours.

#### Western blot analysis

Polyacrylamide gel electrophoresis and Western Blot analysis were conducted according to established methods. The antibodies used were mouse monoclonal antibody clone LMP2-13 directed against 20S proteasome subunit LMP2 (ab78336; Abcam), mouse monoclonal antibody directed against 20S proteasome subunit LMP7 (ab58094; Abcam and 1G7; Novus Biologicals), and mouse monoclonal antibody directed against  $\beta$ -actin (ab6276; Abcam). For detection, a horseradish peroxidase (HRP)-conjugated secondary antibody was used (polyclonal rabbit anti-mouse immunoglobulins/HRP; P 0260; Dako); chemiluminescence detection was carried out with Lumi glow (LumiGLO, Cell signalling technology). The PageRuler Pre-stained Protein Ladder (Fermentas) served as molecular weight standard.

#### Statistical analysis

LMP and TAP allele frequencies were obtained by direct counting. Two-sided Fisher exact test was computed when a table had a cell with an expected frequency of less than 5, otherwise 2-sided exact  $P$  values were used. To calculate  $P$  values, the computer program SPSS (version 10.1.3; SPSS GmbH Software) was used. The  $P$  values are 2-tailed on a 95% significance level. The relative risk (OR) was calculated according to the formula for ORs as:  $OR = (\text{affected patients} \times \text{unaffected controls}) / (\text{unaffected patients} \times \text{affected controls})$ . The 95% CI of the OR was calculated by the computer program SPSS (version 10.1.3). Cell lines CX-1 and WiDr are derivatives of HT-29. Both were excluded from calculations and statistical analyses.

## Results

### Analyses of immunogenetic polymorphisms

Frequencies of TAP alleles and genotypes were defined by sequence analysis (Supplementary Methods and Supplementary Tables S1 and S2), and frequencies of LMP alleles and LMP genotypes by ARMS-PCR in CRC patients ( $n = 174$ ) and in controls ( $n = 165$ ). LMP2 and LMP7 allele frequencies in controls were similar to population frequencies in North American Caucasians (12). No linkage disequilibrium between alleles of TAP, LMP, and MHC class I alleles were observed (data not shown), confirming the solidity of our control panel.

Neither TAP1/TAP2 allele (Supplementary Table S3) nor TAP1/TAP2 genotype frequencies (not shown) of CRC patients differed significantly from the frequencies observed in the control population. However, the immunoproteasome LMP7-K allele was significantly more often found in CRC patients than in controls (24.1% versus 6.1%;  $P = 2.52 \times 10^{-11}$ , Table 1). Frequency deviations seemed to be associated with tumor location (Fig. 1), as the LMP7-K allele was found more often in patients with tumors of the proximal segment (colon) than in patients with tumors of the distal part (rectum; 31.9% versus 15.0%; Table 1). Nevertheless, the association with cancer patients remained significant for all bowel segments analyzed (Table 1). The second allele, LMP7-Q, was significantly more common in control individuals than in CRC patients. The distinct associations between LMP7-K and LMP7-Q alleles and CRC patients became obvious when comparing frequencies of the 3 possible genotype combinations, LMP7-K/K, LMP7-K/Q, and LMP7-Q/Q (Table 2). The LMP7-Q/Q genotype was significantly less frequent in CRC patients than in normal controls. We calculated a 10.0-fold reduced relative risk for colon carcinoma in LMP7-Q/Q homozygous individuals ( $OR = 0.10$ ,  $P = 5.97 \times 10^{-13}$ ; Table 2, Fig. 1). The association of the LMP7-K allele with CRC susceptibility was dominant because CRC patients carried the LMP7-K/Q genotype 3 times more often than control individuals (40.2% versus 12.1%,  $P = 3.88 \times 10^{-9}$ ). The strength of association and relative risk values of the LMP7 genotypes were also significantly influenced by tumor location (Table 2, Fig. 1). Patients with distal tumors, that is, rectal (25.0%) and rectosigmoidal carcinomas (30.0%), carried the LMP7-K/Q genotype nearly twice as often as controls. Patients with more proximal tumor locations, that is, sigmoid carcinomas (41.4%), carried the LMP7-K/Q genotype 3 times more often, and those with the most proximal location, that is, colon carcinomas (52.8%), 4 times more often than controls (colon versus rectum carcinoma,  $P = 5.32 \times 10^{-3}$ ). The maximum OR revealed an 8.10-fold increased relative risk ( $P = 1.10 \times 10^{-11}$ ) for colon cancer in LMP7-K/Q heterozygous individuals (Table 2, Fig. 1).

### Allele and genotype frequencies showed no gender preference

Frequencies of LMP2 alleles in patients did not deviate significantly from frequencies in controls (Table 1). The LMP2-H/H phenotype showed an increased frequency in patients with rectal carcinoma (15.0%; controls 6.7%;  $P > 0.05$ , Table 2).

**Table 1.** *LMP* allele frequencies in colorectal carcinoma patients versus control individuals

<i>LMP</i> alleles	Controls 2n = 330	All CRC patients 2n = 348	Localization of tumors				
			Cecum 2n = 26	Colon 2n = 144	Sigma 2n = 58	Rectosigmoid 2n = 40	Rectum 2n = 80
<i>LMP2-H</i>	90 (27.3%)	103 (29.6%)	9 (34.6%)	47 (32.6%)	11 (19%)	11 (27.5%)	25 (31.3%)
<i>LMP2-R</i>	240 (72.7%)	245 (70.4%)	17 (65.4%)	97 (67.4%)	47 (81.0%)	29 (72.5%)	55 (68.7%)
<i>LMP7-K</i>	20 (6.1%)	84 (24.1%) <sup>a</sup>	4 (15.4%)	46 (31.9%) <sup>b</sup>	14 (24.1%) <sup>c</sup>	8 (20.0%) <sup>d</sup>	12 (15.0%) <sup>e</sup>
<i>LMP7-Q</i>	310 (93.9%)	264 (75.9%)	22 (84.6%)	98 (68.1%)	44 (75.9%)	32 (80.0%)	68 (85.0%)

NOTE: This table gives *LMP* allele frequencies according to tumor localization. In addition, the relative risk (OR) values, significant *P* values, and CIs are stated. The *P* values are valid for both alleles; the OR values are positively associated with *LMP7-K* and inversely associated with *LMP7-Q*.

<sup>a</sup>OR = 4.93 (CI = 2.95–8.25), *P* =  $2.52 \times 10^{-11}$ ,  $\chi^2 = 42.62$  (all colorectal carcinoma patients vs. controls).

<sup>b</sup>OR = 7.28 (CI = 4.11–12.89), *P* =  $1.04 \times 10^{-12}$ ,  $\chi^2 = 56.04$  (colon carcinoma patients vs. controls).

<sup>c</sup>OR = 4.93 (CI = 2.32–10.46), *P* =  $8.31 \times 10^{-5}$ ,  $\chi^2 = 20.16$  (sigma carcinoma patients vs. controls).

<sup>d</sup>OR = 3.87 (CI = 1.58–9.50), *P* =  $5.64 \times 10^{-3}$ ,  $\chi^2 = 9.91$  (rectosigmoid carcinoma patients vs. controls).

<sup>e</sup>OR = 2.73 (CI = 1.28–5.86), *P* =  $1.13 \times 10^{-2}$ ,  $\chi^2 = 7.15$  (rectum carcinoma patients vs. controls).

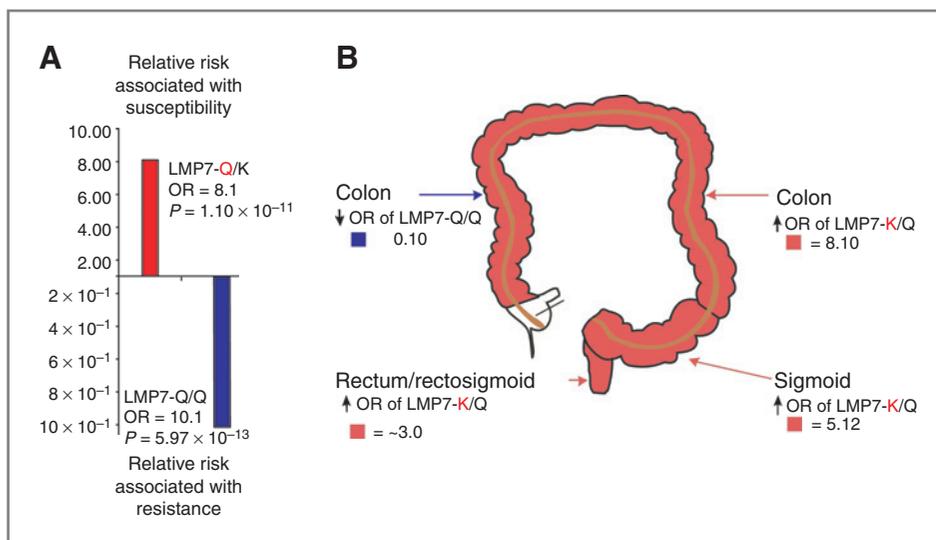
Combined analysis of *LMP2/LMP7* genotypes showed that neither the reduced risk of the *LMP7-Q/Q* genotype nor the increased risk of the *LMP7-K/Q* genotype was significantly altered by combinations with any of the *LMP2* genotypes (Table 3). Therefore, we exclude that *LMP2* could influence susceptibility in rectal carcinomas independently from *LMP7* (Table 2).

To investigate, whether different tumor locations could reflect antigen processing of differentially located microbial factors as suggested for *Helicobacter pylori* in the proximal segment (17) or HPV in the distal segment of the large intestine (3), we used published PCR methods to detect *H. pylori* (18, 19) and HPV (20) in our samples (Supplementary Methods). We could not detect *H. pylori* DNA and only 5 of 100 samples tested were positive for HPV at a lower frequency than reported (3, 20–23).

#### Functional differences between *LMP7-Q/Q* and *LMP7-Q/K* genotypes

Proteolytic activity and peptide generation by the proteasome are altered after IFN- $\gamma$  stimulation by insertion of the *LMP2* and *LMP7* subunits into newly assembling immunoproteasomes (5).

To test for functional differences between *LMP7-Q* and *LMP7-K*, we examined their mRNA expression following IFN- $\gamma$  stimulation. It must be stated, that the *LMP7* polymorphism in this study is located in the presequence of *LMP7* and is therefore absent in the mature protein. The polymorphism can therefore not affect the intrinsic enzymatic activity of *LMP7*, but can only affect the assembly of the immunoproteasome, either by reducing the level of *LMP7* or by interfering with the complex process of assembly, which depends on the presequence. Using probes for



**Figure 1.** Strength of association of *LMP7* alleles with colorectal cancer. A, strength of association of *LMP7* alleles with colorectal cancer given as relative risk values with regard to susceptibility (red column) or resistance (blue column). B, changing values of relative risk shown according to tumor localization.

**Table 2.** LMP genotype frequencies in colorectal carcinoma patients versus control individuals

LMP genotypes	Controls n = 165	All CRC patients n = 174	Localization of tumors				
			Cecum n = 13	Colon n = 72	Sigma n = 29	Rectosigmoid n = 20	Rectum n = 40
LMP2-H/H	11 (6.7%)	16 (9.2%)	2 (15.4%)	6 (8.3%)	1 (3.4%)	1 (5.0%)	6 (15.0%)
LMP2-H/R	68 (41.2%)	71 (40.8%)	5 (38.5)	35 (48.6%)	9 (31.0%)	9 (45.0%)	13 (32.5%)
LMP2-R/R	86 (52.1%)	87 (50.0%)	6 (46.1%)	31 (43.1%)	19 (65.6%)	10 (50.0%)	21 (52.5%)
LMP7-K/K	0 (0%)	7 (4.0%)	0 (0%)	4 (5.5%)	1 (3.4%)	1 (5.0%)	1 (2.5%)
LMP7-Q/K	20 (12.1%)	70 (40.2%) <sup>a</sup>	4 (30.8%)	38 (52.8%) <sup>b</sup>	12 (41.4%) <sup>c</sup>	6 (30.0%) <sup>d</sup>	10 (25.0%) <sup>e,f</sup>
LMP7-Q/Q	145 (87.9%)	97 (55.8%) <sup>g</sup>	8 (69.2%)	30 (41.7%) <sup>h</sup>	16 (55.2%) <sup>i</sup>	13 (65.0%) <sup>j</sup>	29 (72.5%) <sup>k,l</sup>

NOTE: This table gives LMP genotype frequencies according to tumor localization. In addition, the relative risk (OR) values, significant *P* values, and CIs are stated.

<sup>a</sup>OR = 4.88 (CI = 2.80–8.52), *P* = 3.88 × 10<sup>-9</sup>,  $\chi^2$  = 34.31 (all colorectal carcinoma patients vs. controls).

<sup>b</sup>OR = 8.10 (CI = 4.20–15.64), *P* = 1.10 × 10<sup>-11</sup>,  $\chi^2$  = 44.83 (colon carcinoma patients vs. controls).

<sup>c</sup>OR = 5.12 (CI = 2.14–12.27), *P* = 4.23 × 10<sup>-3</sup>,  $\chi^2$  = 15.33 (sigma carcinoma patients vs. controls).

<sup>d</sup>OR = 3.11 (CI = 1.07–9.01), *P* = 4.20 × 10<sup>-2</sup>,  $\chi^2$  = 4.72 (rectosigmoid carcinoma patients vs. controls).

<sup>e</sup>OR = 2.42 (CI = 1.03–5.69), *P* = 4.80 × 10<sup>-2</sup>,  $\chi^2$  = 4.28 (rectum carcinoma patients vs. controls).

<sup>f</sup>OR = 3.35 (CI = 1.43–7.86), *P* = 5.32 × 10<sup>-3</sup>,  $\chi^2$  = 8.10 (colon carcinoma patients vs. rectum carcinoma patients).

<sup>g</sup>OR = 0.17 (CI = 0.10–0.30), *P* = 3.50 × 10<sup>-11</sup>,  $\chi^2$  = 42.81 (all colorectal carcinoma patients vs. controls).

<sup>h</sup>OR = 0.10 (CI = 0.05–0.19), *P* = 5.97 × 10<sup>-13</sup>,  $\chi^2$  = 55.42 (colon carcinoma patients vs. controls).

<sup>i</sup>OR = 0.17 (CI = 0.07–0.41), *P* = 1.10 × 10<sup>-4</sup>,  $\chi^2$  = 18.69 (sigma carcinoma patients vs. controls).

<sup>j</sup>OR = 0.26 (CI = 0.09–0.72), *P* = 1.30 × 10<sup>-2</sup>,  $\chi^2$  = 7.49 (rectosigmoid carcinoma patients vs. controls).

<sup>k</sup>OR = 0.36 (CI = 0.16–0.84), *P* = 2.50 × 10<sup>-2</sup>,  $\chi^2$  = 5.93 (rectum carcinoma patients vs. controls).

<sup>l</sup>OR = 0.27 (CI = 0.12–0.63), *P* = 2.83 × 10<sup>-3</sup>,  $\chi^2$  = 9.81 (colon carcinoma patients vs. rectum carcinoma patients).

exon 3 (E3), the LMP7 mRNA increased only weakly in LMP7-K/K homozygous cell lines, with a mean of 3.84-fold ( $\pm$  2.85), compared with a mean of 10.08-fold ( $\pm$  0.98) in LMP7-Q/Q homozygous cell lines (*P* < 0.05, *U* test; Fig. 2A). The LMP7-K/Q heterozygous cell line showed an intermediate phenotype with an 8.23-fold induction of LMP7-E3. The probes for LMP7 exon 3 detect both of the 2 known splice variants LMP7-E1 (exon 1–exon 3) and LMP7-E2 (exon 2–exon 3; refs. 24, 25; Fig. 2C). Because efficient translation and incorporation has been detected only for LMP7-E2 (26), we also carried out real-time PCR for exon 2 with primers and probes distinguishing the 2 alleles. In addition, we examined the expression of exon 1 (LMP7-E1; Fig. 2A). In LMP7-Q homozygous cell lines, transcripts of LMP7-E1 were inducible with a mean of 3.56-fold ( $\pm$  1.26) and transcripts of LMP7-E2 with a mean of 8.81-fold ( $\pm$  0.61; Fig. 2A). In LMP7-K homozygous cell lines, the induction of LMP7-E1 and LMP7-E2 transcripts was weaker (LMP7-E1 mean 1.1-fold  $\pm$  0.92, LMP7-E2 mean 3.49-fold  $\pm$  2.43, *P* < 0.05, *U* test). Interestingly, the LMP7-K/Q heterozygous cell line showed a differential inducibility of the 2 alleles with a 7.68-fold induction for LMP7-E2-Q and 5.83-fold induction for LMP7-E2-K.

The reduced expression of LMP7 mRNA in LMP7-K/K cells was not associated with reduced induction of HLA-B or of the transporter subunits TAP1 and TAP2 as determined by real-time PCR (Fig. 2B), so that a general defect in the IFN- $\gamma$  induction pathway in this cells can be excluded.

#### Analysis of LMP7 mRNA transcript stability

Because reduced mRNA levels could originate either from reduced transcription or from reduced transcript stability, we analyzed transcript stability with actinomycin D (Fig. 3). The half-life of the LMP7-mRNA was dramatically reduced in the 2 tested LMP7-K/K homozygous cell lines (CaCo2  $t_{1/2}$  = 5.87 minutes, SW948  $t_{1/2}$  = 8.23 minutes), compared with 2 LMP7-Q/Q homozygous cell lines (Colo320DM  $t_{1/2}$  = 28.09 minutes, HT29  $t_{1/2}$  = 38.17 minutes). Therefore, we conclude that the presence of the lysin codon (AAG) at position 49 reduces the LMP7 mRNA stability by 75% (mean  $t_{1/2}$  = 7 minutes) of that observed for the glutamine coding LMP7-Q allele transcript (mean  $t_{1/2}$  = 33 minutes).

To investigate whether the lower level of LMP7 mRNA influences surface HLA class I expression, we analyzed HLA-A, B, C expression of 12 CRC cell lines before and after stimulation with IFN- $\gamma$  (Fig. 4A and B). After stimulation with IFN- $\gamma$ , all LMP7-Q/Q cell lines (Fig. 4C open circles) showed an increased relative MFI of at least 100%, whereas cell lines carrying the LMP7-K allele (Fig. 4C closed or dot-filled circles) showed at best an increase of 20% MFI.

#### LMP7 protein detection in tumor tissue and cell lines

To add another evidence to our analysis, we investigated the amount of Lmp7 protein in tissues of colorectal carcinoma patients. Despite the limited samples, one major problem with this analysis is the fact, that Lmp7 is not a constitutively

**Table 3.** Combinatorial *LMP2/LMP7* genotypes of all CRC patients, colon, and rectum carcinoma patients

<i>LMP</i> genotypes	Controls <i>n</i> = 165	All CRC patients <i>n</i> = 174	Localization of tumors				
			Cecum <i>n</i> = 13	Colon <i>n</i> = 72	Sigma <i>n</i> = 29	Rectosigmoid <i>n</i> = 20	Rectum carcinoma <i>n</i> = 40
<i>LMP2-H/H</i> <i>LMP7-K/K</i>	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)
<i>LMP2-H/R</i> <i>LMP7-K/K</i>	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)
<i>LMP2-R/R</i> <i>LMP7-K/K</i>	0 (0%)	7 (4.0%)	0 (0%)	4 (5.6%)	1 (3.4%)	1 (5.0%)	1 (2.5%)
<i>LMP2-H/H</i> <i>LMP7-Q/Q</i>	10 (6.1%)	11 (6.3%)	1 (7.7%)	3 (4.2%)	1 (3.4%)	1 (5.0%)	5 (12.5%)
<i>LMP2-H/R</i> <i>LMP7-Q/Q</i>	59 (35.8%)	40 (23.0%) <sup>a</sup>	4 (30.8%)	15 (20.8%) <sup>b</sup>	5 (15.2%)	7 (35.0%)	9 (22.5%)
<i>LMP2-R/R</i> <i>LMP7-Q/Q</i>	76 (46.1%)	46 (26.4%) <sup>c</sup>	4 (30.8%)	12 (16.7%) <sup>d</sup>	10 (34.5%)	5 (25.0%)	15 (37.5%)
<i>LMP2-H/H</i> <i>LMP7-Q/K</i>	1 (0.6%)	5 (2.9%)	1 (7.7%)	3 (4.2%)	0 (0%)	0 (0%)	1 (2.5%)
<i>LMP2-H/R</i> <i>LMP7-Q/K</i>	9 (5.4%)	31 (17.8%) <sup>e</sup>	1 (7.7%)	20 (27.8%) <sup>f</sup>	4 (13.8%)	2 (10.0%)	4 (10.0%)
<i>LMP2-R/R</i> <i>LMP7-Q/K</i>	10 (6.1%)	34 (19.5%) <sup>g</sup>	2 (15.4%)	15 (20.8%) <sup>h</sup>	8 (27.6%) <sup>i</sup>	4 (20.0%) <sup>j</sup>	5 (12.5%)

<sup>a</sup>OR = 0.54 (CI = 0.33–0.86),  $P = 1.19 \times 10^{-2}$ ,  $\chi^2 = 6.68$  (CRC vs. controls).

<sup>b</sup>OR = 0.47 (CI = 0.25–0.91),  $P = 3.20 \times 10^{-2}$ ,  $\chi^2 = 5.20$  (colon carcinoma patients vs. controls).

<sup>c</sup>OR = 0.42 (CI = 0.27–0.66),  $P = 1.86 \times 10^{-4}$ ,  $\chi^2 = 14.16$  (CRC vs. controls).

<sup>d</sup>OR = 0.23 (CI = 0.12–0.49),  $P = 1.74 \times 10^{-5}$ ,  $\chi^2 = 18.55$  (colon carcinoma patients vs. controls).

<sup>e</sup>OR = 3.76 (CI = 1.73–8.17),  $P = 6.02 \times 10^{-4}$ ,  $\chi^2 = 12.44$  (CRC vs. controls).

<sup>f</sup>OR = 6.67 (CI = 2.86–15.55),  $P = 5.18 \times 10^{-6}$ ,  $\chi^2 = 23.26$  (colon carcinoma patients vs. controls).

<sup>g</sup>OR = 3.76 (CI = 1.79–7.90),  $P = 2.92 \times 10^{-4}$ ,  $\chi^2 = 13.62$  (CRC vs. controls).

<sup>h</sup>OR = 4.08 (CI = 1.73–9.60),  $P = 1.20 \times 10^{-3}$ ,  $\chi^2 = 11.59$  (colon carcinoma patients vs. controls).

<sup>i</sup>OR = 5.90 (CI = 2.10–16.63),  $P = 1.49 \times 10^{-3}$ ,  $\chi^2 = 13.58$  (sigma carcinoma patients vs. controls).

<sup>j</sup>OR = 3.87 (CI = 1.09–13.78),  $P = 4.90 \times 10^{-2}$ ,  $\chi^2 = 4.96$  (rectosigmoidal carcinoma patients vs. controls).

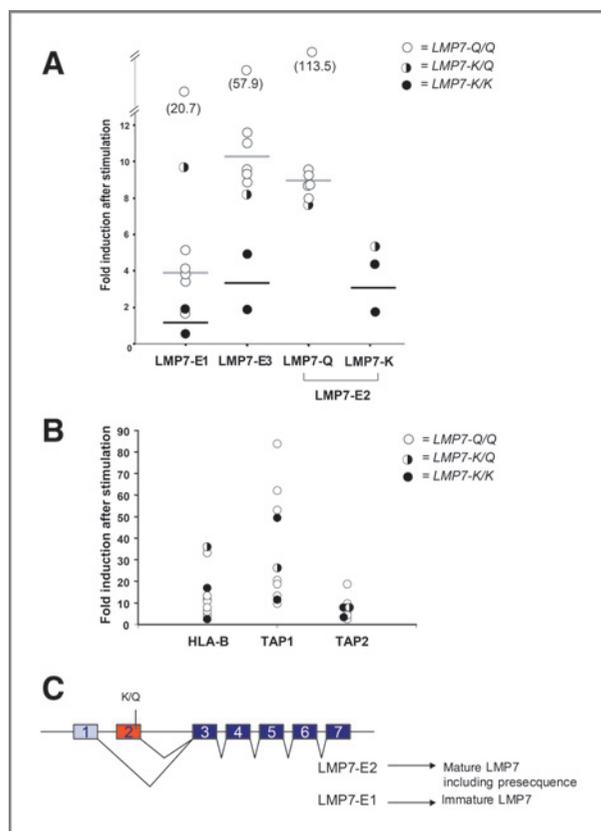
expressed protein, but an IFN $\gamma$  inducible subunit of the immune proteasome. Therefore, we did not expect to detect Lmp7 protein in all tissue samples, but rather we expected only a few specimens to be positive in the Western Blot analysis. Samples not positive for  $\beta$ -actin protein were excluded from the analysis. Out of the 5 tested samples homozygote for *LMP7-K*, none showed a signal with the LMP7-specific antibody, whereas 2 were positive for LMP2. Testing 20 samples of heterozygote patients also showed no positive signal for Lmp7 protein but Lmp2 protein was detected in 6 samples. The samples of the *LMP7-Q/Q* homozygote patients showed a signal for LMP7 in 14 out of 32 tested specimens, and the same samples also showed a signal after re-probing the blot with the antibody directed against LMP2 (Table 4).

By investigating the colorectal cell lines for LMP7 protein (Fig. 4D), we saw results very similar to the mRNA results (Fig. 2). In 4 independent experiments, the *LMP7 Q/Q* (Colo320DM) homozygous cell line increased the amount of LMP7 protein after stimulation up to 28.1-fold, whereas the induction was dramatically reduced to a maximum of 2.0-fold in the *LMP7-K/*

*Q* (WEB2) heterozygous cell line. The homozygous *LMP7-K/K* cell line (SW948) barely produced Lmp7 protein and did not increase the amount after IFN- $\gamma$  stimulation, which is in line with the minimal increase of HLA class I surface expression upon IFN $\gamma$  induction.

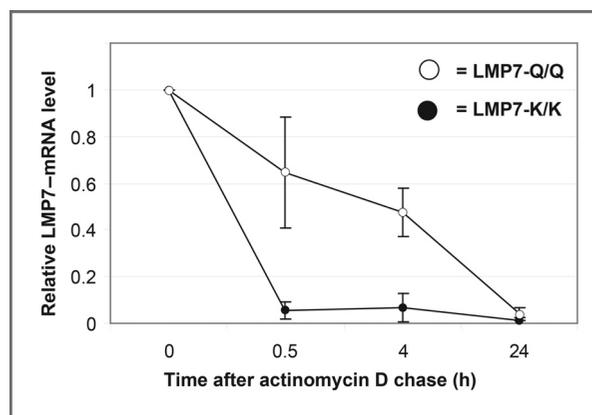
## Discussion

Cancer cells can escape immune recognition by insufficient expression of peptides presented by the MHC because presentation of immunogenic tumor peptides by HLA class I is a prerequisite of a successful antitumor immune responses (3). Efficient expression of peptide–HLA complexes at the cell surface depends on type and quantity of produced and processed peptides. Genetic polymorphisms of immunoproteasome subunits *LMP7* and *LMP2* and of transporter subunits *TAP1* and *TAP2* are documented (11, 12, 27). Association studies indicated a participation of the antigen processing machinery in carcinogenesis, for example, in carcinomas of the esophagus (28) and the cervix (29). In this article, we used



**Figure 2.** Transcript induction after IFN- $\gamma$  treatment in colorectal cancer cell lines carrying the *LMP7-Q/Q*, *LMP7-Q/K*, and *LMP7-K/K* genotype, respectively. Reverse transcriptase real-time PCR analyses of cell lines using G6PD as internal standard and primer probe sets for the dedicated *LMP7*-mRNA transcripts (A) and *HLA-B*, *TAP1*, and *TAP2* (B; x axis). The results are given in fold-induction (y axis) compared with the results of not-stimulated cell lines. A, the bold lines represent the mean values for the cell lines with dedicated genotype. Gray colored lines give the results for *LMP7-Q/Q* carrying cell lines (open circles), black colored lines give the results for *LMP7-K/K* carrying cell lines (filled circles). The half filled circle represents the heterozygous *LMP7-Q/K* cell line WEB2. For the *LMP7-Q/Q* outliers (defined by value above 2 times SD) the fold inductions are given in brackets. Significant differences between *LMP7-K/K* and *LMP7-Q/Q* homozygous cell lines were observed with probes for exon 3 (E3) with a mean of 3.84-fold ( $\pm 2.85$ ) versus a mean of 10.08-fold ( $\pm 0.98$ ) and for exon 2 with a mean of 3.49-fold ( $\pm 2.43$ ) versus an 8.81-fold mean ( $\pm 0.61$ ;  $P < 0.05$  U test). B, as expected, no significant difference of *TAP* and *HLA-B* transcript amounts were observed between the *LMP7-K/K*, *LMP7-Q/Q*, and *LMP7-K/K* carrying cell lines after IFN induction. C, schematic of *LMP7* gene structure, splice variants, and location of allelic variants. The cartoon depicts the *LMP7* gene with its 7 exons (boxes). The exons 1 (light blue) and 2 (red) are used alternatively, resulting in the transcripts *LMP7-E1* and *LMP7-E2*, respectively. Only *LMP7-E2* is transcribed into a mature protein. Exon 2 codes the presequence of *LMP7* that is necessary for the incorporation into the immunoproteasome and is clipped after the assembly of the immunoproteasome. The exons 3 to 7 (dark blue) encode the catalytically active *LMP7* subunit.

colorectal carcinomas to identify susceptibility associated alleles of the antigen processing machinery and to elucidate subsequently functional differences of those alleles. It must be stated, that the *LMP7* polymorphism in this study is located in the presequence of *LMP7* and is therefore absent in the mature

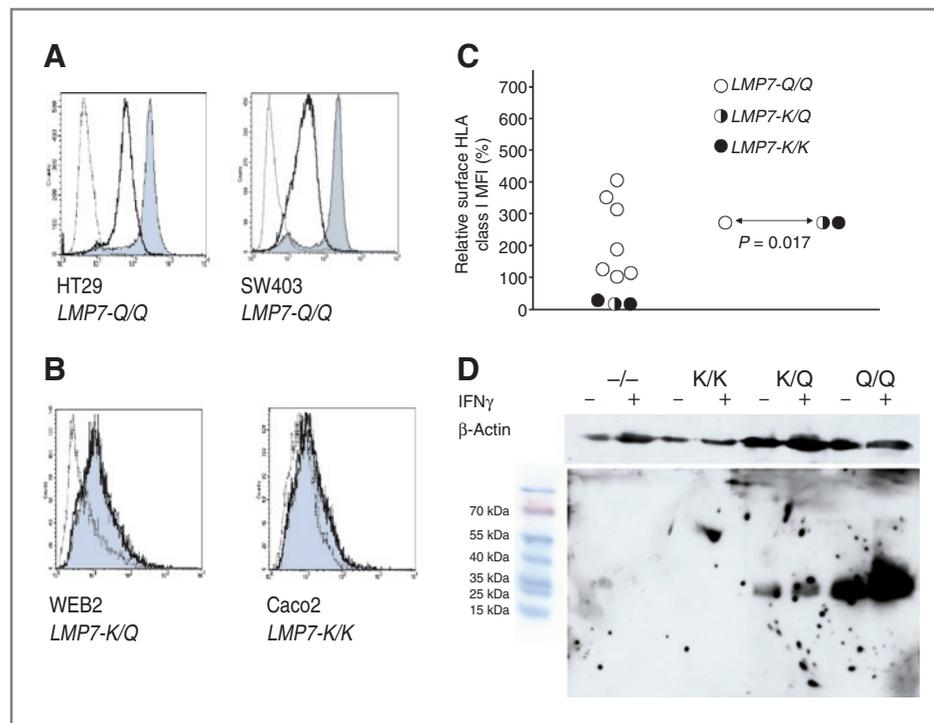


**Figure 3.** Increased mRNA decay of *LMP7-K*. To show the difference in *LMP7-K* and *LMP7-Q* mRNA decay rates, the *LMP7-K/K* cell lines, SW948 and Caco-2, and the *LMP7-Q/Q* cell lines, Colo320DM, were stimulated with IFN- $\gamma$  prior to the addition of actinomycin D. Using 18S-RNA as an internal reference, actinomycin D chase and quantification of *LMP7*-mRNA levels were evaluated as described under Materials and Methods. The decay is given in percentage of transcript amount at time point 0 hour (y axis); the time points are given on the x axis. The mean RNA levels are shown.

protein (see Fig. 2C). The polymorphism can therefore not affect the enzymatic activity of *LMP7*, but can only affect the assembly of the immunoproteasome, either by reducing the level of *LMP7* or by interfering with the complex process of assembly, which depends on the presequence.

*TAP1/TAP2* alleles showed equal frequencies in patients and controls. But the biallelic genetic system of the immunoproteasome subunit *LMP7* showed a strong association with susceptibility to colorectal cancer. Patients with colorectal carcinoma carried the *LMP7-K* allele 3 times more often than control individuals (44.2% versus 12.1%), usually together with the *LMP7-Q* allele (40.2% compared with 12.1% of the controls,  $P = 3.88 \times 10^{-9}$ ; Table 1, Fig. 1). The *LMP7-Q/Q* genotype was underrepresented among patients with colorectal carcinoma (patients 55.8%, controls 87.9%,  $P = 3.50 \times 10^{-11}$ , Table 1). Interestingly, the strength of association between *LMP7-K* and tumor patients was stronger for patients with tumors in the proximal part of the large intestine (colon) and less strong in the distal part (rectum; Table 1, Fig. 1).

The change of strength in disease associations of the *LMP7-K/Q* genotype in different tumor locations could reflect different challenges for the *LMP7-K* allele in processing location-specific tissue or microbial peptides for example *Helicobacter pylori* (17) in the proximal segment or HPV (3) in the distal segment of the large intestine. In addition, it has been shown that the presequence of *LMP7* is a possible target for pathogens (30). Therefore, it is conceivable that certain pathogens of the intestine could disturb immunoproteasome formation. Certain *HLA* alleles and certain HPV types have been identified as main factors in the pathogenesis of cervical and anogenital cancer (3, 22, 31–35), and *Helicobacter pylori* has been implicated in the pathogenesis of gastric cancer and cancer of the colon (17). We were not able to detect *Helicobacter pylori* DNA in any of our tumor samples and found HPV in only 5% of the tumors. This does not exclude a possible impact by a former



**Figure 4.** Fluorescence-activated cell sorting and Western blot analysis of HLA class I expression with and without IFN- $\gamma$  treatment in colorectal cancer cell lines carrying *LMP7-Q/Q*, *LMP7-Q/K*, and *LMP7-K/K*, respectively. Dotted lines represent isotype controls, bold black lines untreated cells, and gray filled curves enclosed by bold lines represent IFN- $\gamma$ -treated cells. Only 2 cell lines for the genotype *LMP7-Q/Q* (A) and 1 cell line each for *LMP7-K/Q* and *LMP7-K/K* genotypes are shown (B); results for the other cell lines were comparable. C, statistical analyses of the relative MFI (%) for IFN- $\gamma$ -treated cells showed a significantly stronger association of *LMP7-Q/Q*-positive cell lines than *LMP7-K/Q* or *LMP7-K/K*-positive cell lines. D, protein induction after IFN- $\gamma$  treatment in colorectal cancer cell lines carrying the *LMP7-Q/Q*, *LMP7-Q/K*, and *LMP7-K/K* genotype, respectively. Western blot analysis of the mature form of LMP7 (bottom) in the presence (+) or absence (-) of IFN- $\gamma$ ;  $\beta$ -actin served as loading control (top). The results are shown for the cell lines T2 (*LMP7-/-*), SW948 (*LMP7-K/K* genotype), WEB2 (*LMP7-Q/Q* genotype), and Colo320DM (*LMP7-Q/Q* genotype), respectively (from left to right). The PageRuler Prestained Protein Ladder (Fermentas) was used as molecular weight standard.

inflammatory infection as evidenced for *Chlamydia trachomatis* and subsequent HPV infections in the development of cervical carcinomas (36).

The strong association observed between *LMP7-K* and colorectal cancer patients prompted us to look for functional consequences of *LMP7-K* versus *LMP7-Q* expression. IFN- $\gamma$  stimulation revealed dramatic differences in the inducibility of *LMP7-K* and *LMP7-Q*. The amount of mRNA increased only weakly in *LMP7-K*-positive cell lines after stimulation with IFN- $\gamma$  (Fig. 2A). In as much as the HLA class I and TAP subunit RNAs were induced in these cells (Fig. 2B), this is likely to be due to the low *LMP7-K* expression and probably a consequence of the significantly reduced mRNA stability of *LMP7-K* (Fig. 3). Chase experiments with actinomycin D indicated that the low *LMP7-K* levels are likely to be a consequence of a reduced half

live (mean  $t_{1/2}$  = 7 minutes) compared with *LMP7-Q* (mean  $t_{1/2}$  = 33 minutes; Fig. 3). *LMP7-K/K* cells were also not able to upregulate surface HLA expression after IFN- $\gamma$  stimulation (Fig. 4).

We predict, that the reduced mRNA stability of *LMP7-K* will lead to reduced amounts of LMP7 proteins, as shown by Western blot analysis (Table 4 and Fig 4D), and therefore to fewer immunoproteasomes. Because the maturation of immunoproteasomes depends on the incorporation of LMP7, impairment in immunoproteasome assembly or maturation could explain the reduced HLA class I surface expression observed in *LMP7-K/Q* cells after IFN- $\gamma$  stimulation. This would alter the processing of microbial as well as of tumor-associated proteins and lead to changes in the peptide repertoire presented by HLA class I molecules. It

**Table 4.** Detection of *LMP2* and *LMP7* protein in CRC patients

	CRC patients	LMP-2 detected	LMP7 detected	$\beta$ -Actin detected
LMP7-K/K	5	2 (40.0%)	0 (0%)	5 (100%)
LMP7-K/Q	20	6 (30.0%)	0 (%)	20 (100%)
LMP7-Q/Q	32	14 (43.7%)	14 (43.7%)	32 (100%)

has to be stated, that although some antigenic peptides are better produced by immunoproteasomes than by regular proteasomes, the opposite is true for other antigenic peptides. It was proposed that deficiency in immunoproteasome expression could diminish the ability of T cells to recognize and destroy tumor cells (37). In a case report, a patient had high numbers of antimelanoma (Melan-A)-specific T cells, but did not destroy the tumor (37). The authors discussed that the "analysis of the proteasome pathway revealed a decreased expression of the subunits of the low molecular weight protein (LMP) 2 and LMP7 as possible explanation for the tumor evasion" (37). Another explanation would be the granzyme levels of the T-cell infiltrates (38). Rudimentary information of T cells would lead to defective immune responses and faster chronic inflammation, a known cofactor in carcinogenesis. LMP7 seems to be involved in inflammatory processes because blocking of LMP7 attenuated progression of experimental arthritis in mice (39). Furthermore, the efficiency of the cross-priming pathway, considered a requisition for the induction of HLA class I-restricted immune responses against tumors, might be impaired as well. The exact mechanism by which *LMP7* alleles influence susceptibility to CRC needs further clarification.

We have shown that alleles of the immunoproteasome subunit LMP7 show distinct reactions upon IFN- $\gamma$  stimulation, displaying fundamental differences of RNA transcript levels, RNA stability, and of pMHC surface expression. It should be examined, whether the failing function of the cancer-associated allele *LMP7-K* could be a target of therapeutic interventions. At present, *LMP7-K* could be used to screen for individuals with the cancer-associated allele, because *LMP7-K* surpasses all other known risk factors associated with sporadic colorectal cancer (40, 41).

### Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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## The *LMP7-K* Allele of the Immunoproteasome Exhibits Reduced Transcript Stability and Predicts High Risk of Colon Cancer

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