The \textit{LMP7-K} allele of the immunoproteasome exhibits reduced transcript stability and predicts high risk of colon cancer

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**Running Title:** *LMP7-K* predicts risk of colon cancer
Abstract

Destruction of cancer cells by cytotoxic T lymphocytes (CTL) depends on immunogenic tumor-peptides generated by proteasomes and presented by 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 (N1=112, N2= 62; controls N=165). High risk of colon cancer was associated with the LMP7-K/Q genotype (OR=8.10, \(P=1.10 \times 10^{-11}\)), 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 interferon (IFN)-\(\gamma\)-stimulation of colon carcinoma cell lines (N=10), followed by analyzes of mRNA expression of HLA class I, TAP1, TAP2 and LMP7, using 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\(\frac{1}{2}\) \(\approx\) 7 min) compared to LMP7-Q (t\(\frac{1}{2}\) \(\approx\) 33 min). Additionally, 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

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processing, followed by reduced peptide-HLA presentation, a crucial factor in the immune response against cancer.
INTRODUCTION

Genetic and environmental factors influence type and growth of malignant tumors. Numerous studies have found associations between particular 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 anti-tumor immune responses requires presentation of immunogenic tumor-peptides by HLA class I and class II molecules (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, while selection and transport is performed by the heterodimeric transporter associated with antigen processing (TAP) (4). Interferon-γ (IFN-γ) 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 compared to 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]) (7, 8)

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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 (CTL) (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 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 and not-disease-associated LMP7 alleles following IFN-γ stimulation by quantifying LMP7 RNA transcripts and analyzing their stability. Finally, we also measured surface expression of HLA molecules in those cell lines.
MATERIAL 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 rectosigmoidal part, and 23 in the rectum. Twenty-five samples of the colon and 11 of the sigma were snap-frozen, all other samples preserved in paraffin. A second independently collected panel from the Munich area consisted of 62 snap frozen carcinoma samples, nine from the cecum, 24 from the colon, 10 from the sigma, two from the rectosigmoidal part, and 17 from the rectum. 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, age 33.6 +/- 9.4 years, for the carcinoma patients male/female ratio was 0.44, age 69.6 +/- 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 (ATCC) and served as positive controls for the LMP-ARMS-PCRs and HPV-specific PCRs, respectively. Colorectal carcinoma lines Colo320DM, SK-Co-1, SW403, Lovo, LS174T, Colo205, HT29, CX1, WiDr, SW948, Caco2 were obtained from the European Type Tissue Type 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% CO₂ in RPMI 1640 (Boehringer Ingelheim), supplemented with 10% FCS. For HLA expression, cancer cell lines were treated for 16 h or 20 h with 200 U/ml recombinant human IFN-γ-1b (Imukin®, Boehringer Ingelheim).

DNA Preparation

Peripheral blood mononuclear cells (PBMCs) of control individuals were isolated by ficoll density gradient centrifugation (PAA Laboratories). Genomic DNA of 1x10⁶ 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 manufacturers instructions. Paraffin embedded tumor samples were deparaffinized with xylene according to the protocol of PeqLab (PeqLab), DNA preparation was performed using 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 et al. and Deng et al. followed the described procedure in order to distinguish LMP2-R and LMP2-H alleles, which have at amino acid (aa) position 60 arginine and histidine, respectively (accession number X66401) (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,

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which enabled us to distinguish between \textit{LMP7-Q} (glutamine) and \textit{LMP7-K} (lysine) differing in aa position 49 of the pro-sequence (accession number Z14982) (12). The allelic controls for both ARMS-PCR protocols were performed by using DNA of the cell lines WT100BIS (\textit{LMP2-H/H / LMP7-Q/Q}) and KAS116 (\textit{LMP2-R/R / LMP7-K/K}) with defined genotypes (16).

**FACS 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 FITC 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 performed 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 x 100%.

**Quantitation of LMP7 RNA by RT-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 manufacturers instruction (Qiagen). For reverse transcription of complete RNA the RevertAid First Strand
cDNA synthesis Kit including random hexamers was chosen, following manufacturers instruction (Fermentas).

All materials for the RT-Real time-PCR were obtained from Applied Biosystems, using the TaqMan Universal PCR Master Mix, and the following pre-designed 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), *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 manufacturers 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 18sRNA) 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 manufacturers instructions), confirming that the absolute value of the slope of log input amount vs. \(C_T\) was below 0.1 (data not shown).
Determination of mRNA Stability by Actinomycin D Treatment

Cell culture cells were stimulated with IFN-γ-1b as described. Following the addition of 50 μg/ml actinomycin D, cells were kept under stimulation condition and samples were taken after 0 min, 30 min, 1h, and 4h.

Western Blot Analysis

Polyacrylamid gele electrophoresis and Western Blot analysis were performed according to established methods. The antibodies used were mouse monoclonal antibody cloneLMP2-13 directed against 20S proteasome subunit LMP2 (ab78336, abcam, Cambridge, UK), mouse monoclonal antibody directed against 20S proteasome subunit LMP7 (ab58094, abcam, Cambridge, UK, and 1G7, Novus Biologicals, Cambridge, UK), and mouse monoclonal antibody directed against β-actin (ab6276, abcam, Cambridge, UK). For detection, a HRP-conjugated secondary antibody was used (polyclonal Rabbit Anti-Mouse Immunoglobulins/HRP; P 0260, Dako, Hamburg, Germany); chemiluminescence detection was performed using Lumi Glow (Lumi Glow, Cell signalling technology). The PageRuler Prestained Protein Ladder (Fermentas, Germany) served as molecular weight standard.
Statistical Analysis

*LMP* and *TAP* allele frequencies were obtained by direct counting. Two-sided Fisher’s exact test was computed when a table had a cell with an expected frequency of less than 5, otherwise two sided exact *P* values were used. To calculate *P* values the computer program SPSS 10.1.3 (SPSS GmbH Software) was used. The *P* values are two tailed on a 95% significance level. The relative risk (OR) was calculated according to the formula for odds ratio: odds ratio (OR) = (affected patients x unaffected controls) / (unaffected patients x affected controls). The 95% confidence interval (CI) of the OR was calculated by the computer program SPSS 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, Supplementary Table S1 & S2 online), 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 IA). Frequency deviations appeared 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 IA). Nevertheless, the association with cancer patients remained significant for all bowel segments analyzed (Table IA). 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 three possible genotype combinations, \( LMP7-K/K, LMP7-K/Q, \) and \( LMP7-Q/Q \) (Table IB). 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 IB, Fig. 1). The association of the \( LMP7-K \) allele with CRC-susceptibility was dominant, since CRC patients carried the \( LMP7-K/Q \) genotype three 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 IB, Fig. 1). Patients with distal tumors, i.e. 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, i.e. sigmoid carcinomas (41.4%), carried the \( LMP7-K/Q \) genotype three times more often, and those with the most proximal location, i.e. colon carcinomas (52.8%), four times more often than controls (colon versus rectum carcinoma, \( P = 5.32 \times 10^{-3} \)). The maximum odds ratio revealed a 8.10-fold increased relative risk (\( P = 1.10 \times 10^{-11} \)) for colon cancer in \( LMP7-K/Q \) heterozygous individuals (Table IB, 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 IA). The \( LMP2-H/H \) genotype showed an increased frequency in patients with rectal carcinoma (15.0%; controls 6.7%; \( P >0.05 \), Table IB). 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 \textit{LMP2} genotypes (Table II). Therefore we exclude that \textit{LMP2} could influence susceptibility in rectal carcinomas independently from \textit{LMP7} (Table IB).

To investigate, whether different tumor locations could reflect antigen processing of differentially located microbial factors as suggested for \textit{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 \textit{H. pylori} (18, 19) and HPV (20) in our samples (Supplementary Methods). We could not detect \textit{H. pylori} DNA and only 5 of 100 samples tested were positive for HPV at a lower frequency than reported (3, 20-23).

\textbf{Functional Differences between \textit{LMP7-Q/Q} and \textit{LMP7-Q/K} Genotypes}

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

To test for functional differences between \textit{LMP7-Q} and \textit{LMP7-K}, we examined their mRNA expression following IFN-\gamma stimulation. It must be stated, that the \textit{LMP7} polymorphism in this study is located in the presequence of \textit{LMP7} and is therefore absent in the mature protein. The polymorphism can therefore not affect the intrinsic enzymatic activity of \textit{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 exon 3 (E3), the LMP7 mRNA increased only weakly in LMP7-K/K homozygous cell lines, with a mean of 3.84-fold (± 2.85), compared to a mean of 10.08-fold (± 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 a 8.23-fold induction of LMP7-E3. The probes for LMP7 exon 3 detect both of the two known splice variants LMP7-E1 (exon 1 - exon 3) and LMP7-E2 (exon 2-exon 3) (24, 25) (Fig. 2C). Since efficient translation and incorporation has been detected only for LMP7-E2 (26), we also performed real-time PCR for exon 2 using primers and probes distinguishing the two 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 (± 1.26) and transcripts of LMP7-E2 with a mean of 8.81-fold (±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 ± 0.92, LMP7-E2 mean 3.49-fold ± 2.43, p<0.05 U-Test). Interestingly, the LMP7-K/Q heterozygous cell line showed a differential inducibility of the two 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-γ-induction pathway in this cells can be excluded.
Analysis of LMP7 mRNA Transcript Stability

Since reduced mRNA levels could originate either from reduced transcription or from reduced transcript stability, we analyzed transcript stability using actinomycin D (Fig. 3). The half-life of the LMP7-mRNA was dramatically reduced in the two tested LMP7-K/K homozygous cell lines (CaCo2 t½ = 5.87 min, SW948 t½ = 8.23 min), compared to two LMP7-Q/Q homozygous cell lines (Colo320DM t½ = 28.09 min, HT29 t½ = 38.17 min). Therefore we conclude, that the presence of the lysin codon (AAG) at position 49 reduces the LMP7 mRNA stability by 75% (mean t½ = 7 min) of that observed for the glutamine coding LMP7-Q allele transcript (mean t½ = 33 min).

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-γ (Fig. 4A & B). After stimulation with IFN-γ, all LMP7-Q/Q cell lines (Fig. 4C open circles) showed an increased relative mean fluorescence intensity (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 expressed protein, but an IFNγ 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 β-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 where 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/LMP7-Q homozygote patients showed in 14 out of 32 tested specimens a signal for LMP7, and the same samples also showed a signal after re-probing the blot with the antibody directed against LMP2 (Table III).

By investigating the colorectal cell lines for LMP7 protein (Fig. 4D) we saw results very similar to the mRNA results (Fig. 2). In four independent experiments, the LMP7 Q/Q (Colo320DM) homozygous cell line increased the amount of LMP7 protein after stimulation up to IFNγ 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-γ stimulation, which is in line with the minimal increase of HLA class I surface expression upon IFNγ induction.
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 are a pre-requisite of a successful anti-tumor 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 paper, we used 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 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 bi-allelic genetic system of the immunoproteasome subunit LMP7 showed a strong association with susceptibility to colorectal cancer. Patients with colorectal

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DISCUSSION
carcinoma carried the \textit{LMP7-K} allele three times more often than control individuals (44.2\% versus 12.1\%), usually together with the \textit{LMP7-Q} allele (40.2\% compared to 12.1\% of the controls $P = 3.88 \times 10^{-9}$) (Table I, Fig. 1). The \textit{LMP7-Q/Q} genotype was underrepresented among patients with colorectal carcinoma (patients 55.8\%, controls 87.9\%, $P = 3.50 \times 10^{-11}$, Table I). Interestingly, the strength of association between \textit{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 I, Fig. 1).

The change of strength in disease associations of the \textit{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 \textit{Helicobacter pylori} (17) in the proximal segment or HPV (3) in the distal segment of the large intestine. Additionally, it has been shown that the pro-sequence 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 human papilloma virus (HPV) types have been identified as main factors in the pathogenesis of cervical and anogenital cancer (3, 22, 31-35), and \textit{Helicobacter pylori} has been implicated in the pathogenesis of gastric cancer and cancer of the colon (17). We were not able to detect \textit{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 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-γ 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-γ (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 t½ = 7 min) compared to *LMP7-Q* (mean t½ = 33 min) (Fig. 3). *LMP7-K/K* cells were also not able to up-regulate surface HLA expression after IFN-γ 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 III and Fig 4D), and therefore to fewer immunoproteasomes. Since 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-γ 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 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 anti melanoma-(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 appears 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 demonstrated, that alleles of the immunoproteasome subunit LMP7 show distinct reactions upon IFN-γ 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, since LMP7-K surpasses all other known risk factors associated with sporadic colorectal cancer (40, 41).
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Online Supplemental Material

The supplemental data include three supplemental tables and one supplemental method and can be found with this article online.

The supplementary methods explain the amplification and sequence analysis of TAP1 and TAP2, as well as the pathogen detection of HPV and H. pylori. Supplementary Table S1 gives the sequence, specificity, and product size of the oligonucleotides used for TAP amplification and sequence analysis. The PCR conditions for TAP amplifications are given in Supplementary Table S2. Supplementary Table S3 shows the TAP allele frequencies in colorectal carcinoma patients according to tumor localization versus control individuals.
REFERENCES


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Table I. *LMP* allele and genotype frequencies in colorectal carcinoma patients versus control individuals

Table I gives *LMP* allele (A) and genotype (B) frequencies according to tumor localization. Additionally, the relative risk (OR) values, significant *P* values, and confidence intervals (CI) are stated.

<table>
<thead>
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<th>Controls</th>
<th>All CRC patients</th>
<th>Localization of tumors</th>
<th>Cecum</th>
<th>Colon</th>
<th>Sigma</th>
<th>Recto-sigmoid</th>
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<td><em>LMP</em> alleles</td>
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<td><em>LMP2-H</em></td>
<td>90 (27.3%)</td>
<td>103 (29.6%)</td>
<td>9 (34.6%)</td>
<td>47 (32.6%)</td>
<td>11 (19%)</td>
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<tr>
<td><em>LMP2-R</em></td>
<td>240 (72.7%)</td>
<td>245 (70.4%)</td>
<td>17 (65.4%)</td>
<td>97 (67.4%)</td>
<td>47 (81.0%)</td>
<td>29 (72.5%)</td>
<td>55 (68.7%)</td>
<td></td>
</tr>
<tr>
<td><em>LMP7-K</em></td>
<td>20 (6.1%)</td>
<td>84 (24.1%)</td>
<td>4 (15.4%)</td>
<td>46 (31.9%)</td>
<td>14 (24.1%)</td>
<td>8 (20.0%)</td>
<td>12 (15.0%)</td>
<td></td>
</tr>
<tr>
<td><em>LMP7-Q</em></td>
<td>310 (93.9%)</td>
<td>264 (75.9%)</td>
<td>22 (84.6%)</td>
<td>98 (68.1%)</td>
<td>44 (75.9%)</td>
<td>32 (80.0%)</td>
<td>68 (85.0%)</td>
<td></td>
</tr>
</tbody>
</table>

The *P* values are valid for both alleles; the OR values are positively associated with *LMP7-K*, and inversely associated with *LMP7-Q*.

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

*b* OR = 7.28 (CI 4.11-12.89), \( P = 1.04 \times 10^{-12} \), \( \chi^2 = 56.04 \) (colon carcinoma patients vs. controls)
c OR = 4.93 (CI 2.32-10.46), \( P = 8.31 \times 10^{-5} \chi^2 = 20.16 \) (sigma carcinoma patients vs. controls)

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

e OR = 2.73 (CI 1.28-5.86), \( P = 1.13 \times 10^{-2} \chi^2 = 7.15 \) (rectum carcinoma patients vs. controls)
### Table IB. LMP genotype frequencies

<table>
<thead>
<tr>
<th>LMP genotypes</th>
<th>Controls $n = 165$</th>
<th>All CRC patients $n = 174$</th>
<th>Localization of tumors</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Cecum $n = 13$</td>
<td>Colon $n = 72$</td>
<td>Sigma $n = 29$</td>
</tr>
<tr>
<td>LMP2-H / H</td>
<td>11 (6.7%)</td>
<td>16 (9.2%)</td>
<td>2 (15.4%)</td>
</tr>
<tr>
<td>LMP2-H / R</td>
<td>68 (41.2%)</td>
<td>71 (40.8%)</td>
<td>5 (38.5)</td>
</tr>
<tr>
<td>LMP2-R / R</td>
<td>86 (52.1%)</td>
<td>87 (50.0%)</td>
<td>6 (46.1%)</td>
</tr>
<tr>
<td>LMP7-K / K</td>
<td>0 (0%)</td>
<td>7 (4.0%)</td>
<td>0 (0%)</td>
</tr>
<tr>
<td>LMP7-Q / K</td>
<td>20 (12.1%)</td>
<td>70 (40.2%)</td>
<td>4 (30.8%)</td>
</tr>
<tr>
<td>LMP7-Q / Q</td>
<td>145 (87.9%)</td>
<td>97 (55.8%)</td>
<td>8 (69.2%)</td>
</tr>
</tbody>
</table>

- **OR** = 4.88 (CI 2.80-8.52), $P = 3.88 \times 10^{-9} \chi^2 = 34.31$ (all colorectal carcinoma patients vs. controls)
- **OR** = 8.10 (CI 4.20-15.64), $P = 1.10 \times 10^{-11} \chi^2 = 44.83$ (colon carcinoma patients vs. controls)
- **OR** = 5.12 (CI 2.14-12.27), $P = 4.23 \times 10^{-3} \chi^2 = 15.33$ (sigma carcinoma patients vs. controls)
- **OR** = 3.11 (CI 1.07-9.01), $P = 4.20 \times 10^{-2} \chi^2 = 4.72$ (rectosigmoid carcinoma patients vs. controls)
e OR = 2.42 (CI 1.03-5.69), \( P = 4.80 \times 10^{-2} \) \( \chi^2 = 4.28 \) (rectum carcinoma patients vs. controls)

f OR = 3.35 (CI 1.43-7.86), \( P = 5.32 \times 10^{-3} \) \( \chi^2 = 8.10 \) (colon carcinoma patients vs. rectum carcinoma patients)

g OR = 0.17 (CI 0.10-0.30), \( P = 3.50 \times 10^{-11} \) \( \chi^2 = 42.81 \) (all colorectal carcinoma patients vs. controls)

h OR = 0.10 (CI 0.05-0.19), \( P = 5.97 \times 10^{-13} \) \( \chi^2 = 55.42 \) (colon carcinoma patients vs. controls)

i OR = 0.17 (CI 0.07-0.41), \( P = 1.10 \times 10^{-4} \) \( \chi^2 = 18.69 \) (sigma carcinoma patients vs. controls)

k OR = 0.26 (CI 0.09-0.72), \( P = 1.30 \times 10^{-2} \) \( \chi^2 = 7.49 \) (rectosigmoid carcinoma patients vs. controls)

l OR = 0.36 (CI 0.16-0.84), \( P = 2.50 \times 10^{-2} \) \( \chi^2 = 5.93 \) (rectum carcinoma patients vs. controls)

m OR = 0.27 (CI 0.12-0.63), \( P = 2.83 \times 10^{-3} \) \( \chi^2 = 9.81 \) (colon carcinoma patients vs. rectum carcinoma patients)
Table II. Combinatorial LMP2 / LMP7 genotypes of all CRC patients, colon and rectum carcinoma patients.

<table>
<thead>
<tr>
<th>LMP genotypes</th>
<th>Controls ( n = 165 )</th>
<th>All CRC patients ( n = 174 )</th>
<th>Caecum ( n = 13 )</th>
<th>Colon ( n = 72 )</th>
<th>Sigma ( n = 29 )</th>
<th>Recto-sigmoid ( n = 20 )</th>
<th>Rectum carcinoma ( n = 40 )</th>
</tr>
</thead>
<tbody>
<tr>
<td>LMP2-H / H</td>
<td>0 (0%)</td>
<td>0 (0%)</td>
<td>0 (0%)</td>
<td>0 (0%)</td>
<td>0 (0%)</td>
<td>0 (0%)</td>
<td>0 (0%)</td>
</tr>
<tr>
<td>LMP7-K / K</td>
<td>0 (0%)</td>
<td>0 (0%)</td>
<td>0 (0%)</td>
<td>0 (0%)</td>
<td>0 (0%)</td>
<td>0 (0%)</td>
<td>0 (0%)</td>
</tr>
<tr>
<td>LMP2-H / R</td>
<td>0 (0%)</td>
<td>0 (0%)</td>
<td>0 (0%)</td>
<td>0 (0%)</td>
<td>0 (0%)</td>
<td>0 (0%)</td>
<td>0 (0%)</td>
</tr>
<tr>
<td>LMP7-K / K</td>
<td>0 (0%)</td>
<td>7 (4.0%)</td>
<td>0 (0%)</td>
<td>4 (5.6%)</td>
<td>1 (3.4%)</td>
<td>1 (5.0%)</td>
<td>1 (2.5%)</td>
</tr>
<tr>
<td>LMP2-R / R</td>
<td>10 (6.1%)</td>
<td>11 (6.3%)</td>
<td>1 (7.7%)</td>
<td>3 (4.2%)</td>
<td>1 (3.4%)</td>
<td>1 (5.0%)</td>
<td>5 (12.5%)</td>
</tr>
<tr>
<td>LMP7-Q / Q</td>
<td>59 (35.8%)</td>
<td>40 (23.0%)</td>
<td>4 (30.8%)</td>
<td>15 (20.8%)</td>
<td>5 (15.2%)</td>
<td>7 (35.0%)</td>
<td>9 (22.5%)</td>
</tr>
<tr>
<td>LMP2-R / R</td>
<td>76 (46.1%)</td>
<td>46 (26.4%)</td>
<td>4 (30.8%)</td>
<td>12 (16.7%)</td>
<td>10 (34.5%)</td>
<td>5 (25.0%)</td>
<td>15 (37.5%)</td>
</tr>
<tr>
<td>LMP7-Q / K</td>
<td>1 (0.6%)</td>
<td>5 (2.9%)</td>
<td>1 (7.7%)</td>
<td>3 (4.2%)</td>
<td>0 (0%)</td>
<td>0 (0%)</td>
<td>1 (2.5%)</td>
</tr>
<tr>
<td>LMP2-R / R</td>
<td>9 (5.4%)</td>
<td>31 (17.8%)</td>
<td>1 (7.7%)</td>
<td>20 (27.8%)</td>
<td>4 (13.8%)</td>
<td>2 (10.0%)</td>
<td>4 (10.0%)</td>
</tr>
<tr>
<td>LMP7-Q / K</td>
<td>10 (6.1%)</td>
<td>34 (19.5%)</td>
<td>2 (15.4%)</td>
<td>15 (20.8%)</td>
<td>8 (27.6%)</td>
<td>4 (20.0%)</td>
<td>5 (12.5%)</td>
</tr>
</tbody>
</table>

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

\(^b\) OR = 0.47 (CI 0.25-0.91), \( P = 3.20 \times 10^{-2} \chi^2 = 5.20 \) (colon carcinoma patients vs. controls)
c OR = 0.42 (CI 0.27-0.66), \( P = 1.86 \times 10^{-4} \chi^2 = 14.16 \) (CRC vs. controls)

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

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

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

\[ \text{CRC vs. controls} \]

\[ \chi^2 = 12.44 \]

\[ \text{CRC vs. controls} \]

\[ \chi^2 = 23.26 \]

\[ \text{CRC vs. controls} \]

\[ \chi^2 = 13.62 \]

\[ \text{CRC vs. controls} \]

\[ \chi^2 = 11.59 \]

\[ \text{CRC vs. controls} \]

\[ \chi^2 = 13.58 \]

\[ \text{CRC vs. controls} \]

\[ \chi^2 = 4.96 \]

\[ \text{CRC vs. controls} \]

\[ \chi^2 = 4.96 \]
Table III. Detection of LMP2 and LMP7 protein in CRC patients.

<table>
<thead>
<tr>
<th>CRC patients</th>
<th>LMP-2 detected</th>
<th>LMP7 detected</th>
<th>ß-Actin detected</th>
</tr>
</thead>
<tbody>
<tr>
<td>LMP7-K/K</td>
<td>5</td>
<td>2 (40.0%)</td>
<td>0 (0%)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0 (0%)</td>
<td>5 (100%)</td>
</tr>
<tr>
<td>LMP7-K/Q</td>
<td>20</td>
<td>6 (30.0%)</td>
<td>0 (%)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>20 (100%)</td>
<td></td>
</tr>
<tr>
<td>LMP7-Q/Q</td>
<td>32</td>
<td>14 (43.7%)</td>
<td>14 (43.7%)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>32 (100%)</td>
<td></td>
</tr>
</tbody>
</table>
Figure legends

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.

Figure 2 Transcript induction after IFN-γ treatment in colorectal cancer cell lines carrying the *LMP7-Q/Q*, *LMP7-Q/K*, and *LMP7-K/K* genotype, respectively

RT-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 to 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 standard deviation) the fold-inductions are given in brackets. Significant differences between *LMP7-K/K* and *LMP7-Q/Q* homozygous cell lines were observed using probes for exon 3 (E3) with a mean of 3.84-fold (± 2.85) vs. a mean of 10.08-fold (± 0.98), and for exon 2 with a mean of 3.49-fold (± 2.43) vs. a 8.81-fold mean (±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 immuno-proteasome and is clipped after the assembly of the immunoproteasome. The exons 3 to 7 (dark blue) encode the catalytically active LMP7 subunit.

**Figure 3 Increased mRNA decay of LMP7-K**

To demonstrate 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-γ prior to the addition of actinomycin D. Using 18S-RNA as internal reference, actinomycin D chase and quantification of LMP7-mRNA levels were evaluated as described under “Method”. The decay is given in percent of transcript amount at time point 0h (y-axis), the times points are given on the x-axis. The mean RNA levels are shown.

**Figure 4 FACS and Western Blot analysis of HLA class I expression with and without IFN-γ 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-γ treated cells. Only two cell
lines for the genotype LMP-7 Q/Q (A), and one cell line each for LMP-7 K/Q and LMP-7 K/K genotypes are shown (B), results for the other cells lines were comparable. (C) Statistical analyses of the relative mean fluorescence intensity (% MFI) for IFN-γ treated cells showed a significant stronger association of LMP-7 Q/Q positive cell lines than LMP-7 K/Q or LMP-7 K/K positive cell lines. (D) Protein induction after IFN-γ 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-γ; β-actin served as loading control (top). The results are shown for the cell lines T2 (LMP7 -/-), SW948 (LMP7-K/K genotype, WEB2 (LMP7-Q/K genotype), and Colo320DM (LMP7-Q/Q genotype), respectively (from left to right). The PageRuler Prestained Protein Ladder (Fermentas, Germany) was used as molecular weight standard.
Figure 1

A

Relative risk associated with susceptibility

LMP7-Q/K
OR = 6.1  
$P = 1.10 \times 10^{-11}$

LMP7-Q/Q
OR = 10.1  
$P = 5.97 \times 10^{-12}$

B

Colon
OR of LMP7-Q/Q  
$= 0.10$

Colon
OR of LMP7-K/Q  
$= 8.10$

Rectum/rectosigmoid
OR of LMP7-K/Q  
$= 3.0$

Sigmoid
OR of LMP7-K/Q  
$= 5.12$
Figure 2

A

B

C

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Figure 4

A

HT29  LMP7-Q/Q
SW403  LMP7-Q/Q

B

WEB2  LMP7-K/Q
Caco2  LMP7-K/K

C

Relative surface HLA class I expression (%)

D

IFNγ

β-actin

70 kDa  55 kDa  40 kDa  35 kDa  25 kDa  15 kDa
The LMP7-K allele of the immunoproteasome exhibits reduced transcript stability and predicts high risk of colon cancer

Barbara Fellerhoff, Songhai Gu, Barbara Laumbacher, et al.

Cancer Res Published OnlineFirst October 28, 2011.

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