NLRC5/MHC class I transactivator is a target for immune evasion in cancer

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Cancer cells develop under immune surveillance, thus necessitating immune escape for successful growth. Loss of MHC class I expression provides a key immune evasion strategy in many cancers, although the molecular mechanisms remain elusive. MHC class I transactivator (CITA), known as "NLRC5" [NOD-like receptor (NLR) family, caspase recruitment (CARD) domain containing 5], has recently been identified as a critical transcriptional coactivator of MHC class I gene expression. Here we show that the MHC class I transactivation pathway mediated by CITA/NLRC5 constitutes a target for cancer immune evasion. In all the 21 tumor types we examined, *NLRC5* expression was highly correlated with the expression of MHC class I, with cytotoxic T-cell markers, and with genes in the MHC class I antigen-presentation pathway, including *LMP2*/*LMP7*, *TAP1*, and β2-microglobulin. Epigenetic and genetic alterations in cancers, including promoter methylation, copy number loss, and somatic mutations, were most prevalent in *NLRC5* among all MHC class I-related genes and were associated with the impaired expression of components of the MHC class I pathway. Strikingly, *NLRC5* expression was significantly associated with the activation of $CD8^+$ cytotoxic T cells and patient survival in multiple cancer types. Thus, NLRC5 constitutes a novel prognostic biomarker and potential therapeutic target of cancers.

MHC class I | CITA | cancer | immune evasion | NLRC5

During cancer progression, neoplastic cells accumulate numerous mutations that constitute potentially immunogenic neo-epitopes. Thus, most tumors concurrently need to use mechanisms that enable escape from immune surveillance for successful growth and progression (1). It has been demonstrated that cancer cells use multiple strategies of immune evasion, including increased resistance to cytotoxic T-cell killing, induction of anergy in activated T cells, elimination of effector T cells, recruitment of regulatory immune cell subsets, and reduced recognition of tumor-associated antigens by effector T cells (2). Impaired MHC class I-mediated antigen presentation is a major immune evasion mechanism in cancer (3, 4), with MHC class I loss reported in cervical cancer (92%) (5), penile cancer (80%) (6), breast cancer (71%) (7), nonsmall cell lung cancer (64%) (8), and esophageal squamous cell carcinoma (67%) (9), among others. Although a number of mechanisms have been described for HLA loss, including the loss of heterozygosity, HLA gene methylation, nonsense/missense mutations, and loss of *TAP1/2* or β2-microglobulin (*B2M*), the dominant underlying molecular mechanism seems to reside at the transcriptional level (10). Transcriptional regulation of MHC class I genes remained largely undefined until the recent discovery of CITA (MHC class I transactivator), known as NLRC5 [NOD-like receptor (NLR) family, caspase recruitment (CARD) domain containing 5] (11, 12). NLRC5 is an IFN-γ–inducible nuclear protein (13–15) that specifically associates with and activates promoters of MHC class I genes by generating a CITA enhanceosome complex with other transcription factors (14, 16, 17). A striking feature of CITA/NLRC5 is that it does not solely induce MHC class I genes but also activates other critical genes involved in the MHC class I antigen-presentation pathway, including the immunoproteasome component *LMP2* (*PSMB9*), peptide transporter *TAP1*, and *B2M* (14, 17), thus regulating most of the key components in the MHC class I antigen-presentation machinery. *Nlrc5*-deficient mice exhibit impaired constitutive and inducible expression of MHC class I genes in vivo (18–22). In addition, *Nlrc5*-deficient cells display an impaired ability to elicit $CD8^+$ T-cell activation, as evidenced by impaired IFN-γ production and diminished cytolytic activity (18, 19, 21).

Results

Expression of *NLRC5* and MHC Class I Genes Is Correlated in Human Cancers. Because of the prominent role of NLRC5 in orchestrating the expression of MHC class I and class I-related genes, we examined gene-expression profiles of biopsy samples from the cohort of 7,747 solid cancer patients in The Cancer Genome Atlas (TCGA) database. The expression of *HLA-B* was highly correlated with the level of *NLRC5* expression in the entire cohort ($r_s = 0.753$) (Fig. 1*A*). Correlation analysis for gene expression among 14 cancer types demonstrated that *HLA-B* and *NLRC5* expression showed high positive correlation ($r_s > 0.70$) in nine cancer types and intermediate positive correlation ($r_s > 0.50$) in five cancer types (Fig. 1 *B* and *C*), with the highest correlation observed in melanoma. In addition to *HLA-B*, the expression of *HLA-A*, *HLA-C*, *B2M*, *LMP2*, *LMP7* (*PSMB8*), and *TAP1* was also highly correlated with *NLRC5* expression in melanoma and other cancers (Fig. 1*D* and Fig. S1*A*).

Significance

Tumor antigen presentation to $CD8^+$ T cells by MHC class I molecules is crucial for immune responses against cancers, whereas the loss of MHC class I is a common immune evasion strategy used by cancers. However, the molecular mechanisms leading to MHC class I deficiency have remained poorly defined. We demonstrate here that MHC class I transactivator (CITA)/NOD-like receptor (NLR) family, caspase recruitment (CARD) domain containing 5 (NLRC5) is a major target for cancer immune evasion. Reduced expression of MHC class I and related genes in cancer is frequently associated with genetic and epigenetic changes in *NLRC5*. The reduced *NLRC5* expression is linked to impaired $CD8^+$ T-cell activation and poor patient prognosis. These data indicate that CITA/NLRC5 is a novel prognostic marker and potential therapeutic target of cancers.

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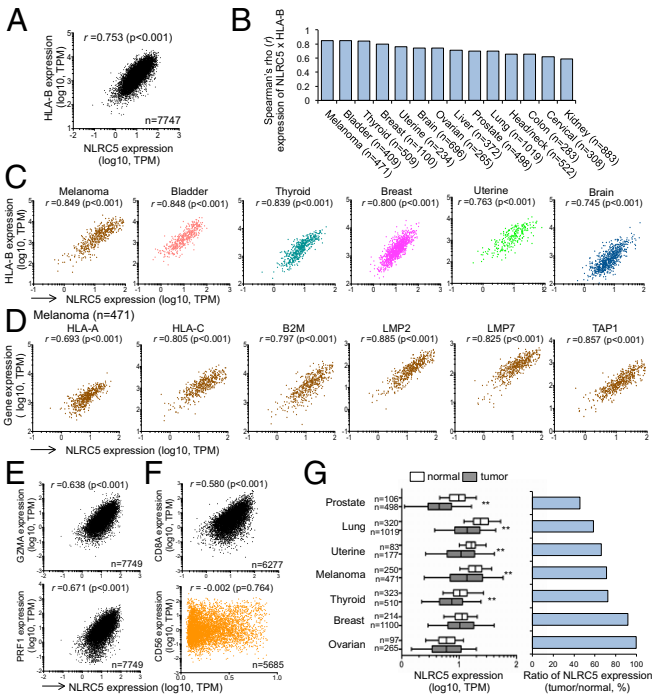

Fig. 1. Expressions of *NLRC5* and MHC class I genes are positively correlated. (*A*) Scatter plots for the expression of *NLRC5* [*x* axis; log10 values in transcripts per million (TPM)] and *HLA-B* (*y* axis; log10 values in TPM) in 16 tumor types (n = 7,747). (*B*) Spearman rank correlation coefficients between the expression of *NLRC5* and *HLA-B*. Fourteen representative tumor types carrying at least 100 samples are shown. (*C*) Scatter plots for the expression of *NLRC5* and *HLA-B* in six tumor types showing high correlation coefficients. (*D*) Scatter plots for the expression of *NLRC5* and other MHC class I-related genes in melanoma that have the highest correlation coefficients in *B*. (*E*) Scatter plots for the expression of *NLRC5* and *GZMA* or *PRF1* in 16 tumor types (n = 7,749). (*F*) Scatter plots for the expression of *NLRC5* and *CD8A* in 16 tumor types (n = 6,277) or *CD56* in 15 tumor types (n = 5,685). Pairwise correlations in *A*–*F* were calculated using the Spearman's ranked correlation test; *r*, Spearman rho coefficient. (*G*, *Left*) *NLRC5* expression in indicated normal and tumor tissues. The bar inside the box corresponds to the median; the box corresponds to the 25th–75th percentiles, and the error bars indicate the confidence interval (fifth–95th percentile). Statistical significance was determined by the Mann–Whitney test: $^{**}P < 0.01$. (*Right*) The ratio of *NLRC5* expression level in tumor and normal tissues.

Because CITA/NLRC5-mediated MHC class I expression is crucial for optimal activation and cytolytic activity of $CD8^+$ T cells (18, 19), we next examined the expression level of perforin (*PRF1*) or granzyme A (*GZMA*), which are known to be associated with cytotoxic T-cell activity in cancer tissues (23). Indeed, the cohort of 16 solid cancer etiologies revealed a significant positive correlation between *NLRC5* expression and *PRF1* or *GZMA* (Fig. 1*E* and Fig. S1*B*). Although *PRF1* and *GZMA* are expressed in both activated $CD8^+$ T cells and natural killer (NK) cells, *NLRC5* expression was correlated only with *CD8A* but not with the NK cell marker *CD56* (Fig. 1*F* and Fig. S1*C*). These data indicate that *NLRC5* expression in cancer tissues is critical for inducing $CD8^+$ T-cell–dependent cytotoxic activity, likely through the induction of MHC class I expression. Despite the critical function of NLRC5 for MHC class I-dependent immune responses, there are likely to be aberrant mechanisms which may reduce *NLRC5* expression in cancers, as indicated by decreased *NLRC5* expression in multiple cancer types compared with normal tissues (Fig. 1*G*). Three cancer types with higher expression of *NLRC5* seemed to be exceptions (Fig. S1*D*), perhaps because of a high inflammatory state (hepatocellular carcinoma and colorectal carcinoma) (24, 25) or increased infiltration of hematopoietic cells characterized by high *CD45* expression (brain tumors) (Fig. S1*E*).

Preferential Methylation of NLRC5 in Cancer Is Associated with Impaired Cytotoxic T-Lymphocyte Activity. Epigenetic changes in cancer cells represent an important mechanism to alter gene expression in favor of cancer growth and immune evasion (26). Abnormal methylation of CpG islands in promoter regions can transcriptionally suppress genes that are unfavorable for cancer growth (27). Treatment of various cancer cell lines with a DNA-methylation inhibitor, 5-azacitidine (5-Aza), resulted in the up-regulation of *NLRC5* and *HLA-B* expression, suggesting that methylation of the *NLRC5* promoter might play a role in the loss of MHC class I expression in cancer (Fig. 2*A*). Therefore, the level of DNA methylation at a CpG island in the *NLRC5* promoter in various cancer types was quantified using a methylation-specific probe (Fig. 2*B*). Methylation of the *NLRC5* promoter was observed at higher frequency in multiple cancers than in the corresponding normal tissues (excluding prostate, thyroid, and kidney, where high methylation was observed even in normal tissues) (Fig. 2*C* and Fig. S2*A*). Furthermore, analysis of biopsy samples from 6,523 solid cancer patients revealed that methylation of the *NLRC5* promoter was negatively correlated with *NLRC5* expression (r_s = −0.585) (Fig. 2*D*). Suppression of *NLRC5* expression by promoter methylation was observed in all 13 cancer types that we examined; an intermediate negative correlation (r_s = −0.50 to −0.70) was found in five cancer types, and a low negative correlation (r_s = −0.30 to −0.50) was found in eight cancer types (Fig. S2 *B* and *C*). Moreover, the methylation of the *NLRC5* promoter was negatively correlated with the expression of *HLA-B* in all cancer types to various degrees (Fig. 2*E* and Fig. S2*C*). *NLRC5* promoter methylation also was negatively correlated with the expression of *HLA-A*, *HLA-C*, *B2M*, *LMP2*, *LMP7*, and *TAP1* in melanoma and other cancers (Fig. 2*E* and Fig. S2*D*). Reduced expression of MHC class I genes was specifically correlated with *NLRC5* methylation because methylation of the promoter for *CIITA*, a master transcriptional activator of MHC class II genes, did not correlate with the expression of *HLA-B* or other class I-related genes in the entire cancer cohort or in melanoma (Fig. 2*F* and Fig. S2*E*). Strikingly, *NLRC5* methylation was negatively correlated with *CD8A, GZMA*, and *PRF1* but not with *CD56* (Fig. 2 *G* and *H* and Fig. S2 *F* and *G*). These data suggest that methylation of *NLRC5* in cancer cells results in the transcriptional suppression of *NLRC5*, leading to reduced expression of MHC class I genes and evasion of $CD8^+$ cytotoxic T-cell–dependent antitumor activity. Because HLA gene methylation also has been reported in cancer cells (10), the methylation level of the *NLRC5* promoter was compared with that of other MHC class I and related genes. Although various degrees of *NLRC5* methylation were observed in all the different cancer types examined (Fig. S2*H*), the DNA methylation was most severe in *NLRC5* among all class I-related genes tested in entire cancer cohort (Fig. 2*I*). Moreover, methylation of the *NLRC5* promoter exhibited the most effective gene suppression among all class I-related genes, because the negative correlation between DNA methylation and gene expression was more prominent for *NLRC5* than for other MHC class I-related genes (Fig. 2 *D*, *J*, and *K* and Fig. S2*I*). Taken together, these data suggest that the methylation of *NLRC5*, but not of other MHC class I genes, is used selectively in various cancers as an immune evasion strategy for efficient suppression of the MHC class I pathway.

Copy Number Loss of *NLRC5* Is Associated with Reduced MHC Class I Gene Expression. Changes in somatic gene copy number (CN) are frequently observed in cancer cells and are associated with altered gene-expression levels (28, 29). The analysis of CN in the cohort of 7,730 cancer patients showed that all cancer types carry alterations in CN of the *NLRC5* gene. CN loss (CN = 0 or 1) was observed in 28.6% of all cancer patients, with the highest frequency (72.2%) in ovarian cancer patients (Fig. 3*A*). Remarkably, among MHC class I and related genes across the entire cancer cohort and in ovarian cancer, the frequency of CN loss was highest for *NLRC5*, followed by *B2M* (Fig. 3*B* and Fig. S3*A*), again indicating that *NLRC5* is a preferential target for cancer

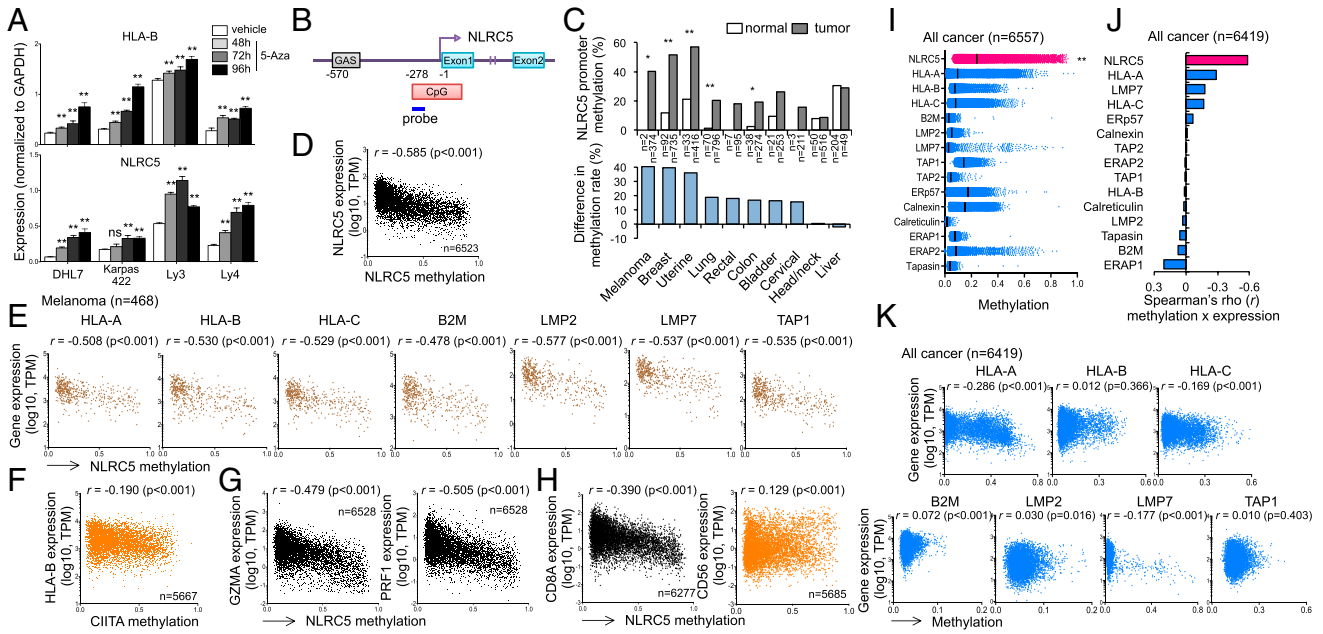

Fig. 2. Preferential DNA methylation in the *NLRC5* promoter in cancer cells is associated with impaired MHC class I-dependent cytotoxic T-cell activity. (*A*) Indicated cancer cell lines were treated with 3 μM of 5-Aza for the indicated time periods, and *NLRC5* and *HLA-B* expression was quantified by quantitative PCR. Data are representative of three independent experiments and are shown as means ± SD. Statistical significance was determined by the paired *t* test: $^{**}P < 0.01$. (*B*) Schematic representation of the methylation-specific probe on the *NLRC5* promoter region. The *NLRC5* promoter has a CpG island of ∼578 bp starting at position −278. To examine the methylation status of the *NLRC5* promoter, a methylation-specific probe (cg16411857, blue line) on the CpG island was used. The transcription start site is indicated as −1; the STAT1-binding site GAS is at −570. (*C*, *Upper*) The methylation rate of the *NLRC5* promoter in 10 indicated cancer types and normal tissues. (*Lower*) The difference in the *NLRC5* promoter methylation rate in tumor and normal tissues. A β value over 0.3 was considered as methylated. Statistical significance was determined by the χ^2 test: $^{*}P < 0.05$; $^{**}P < 0.01$. (*D*) Scatter plots showing the expression of *NLRC5* (*y* axis; log10 values in TPM) and the methylation level of the *NLRC5* promoter (*x* axis; β values) in 15 tumor types ($n = 6{,}523$). (*E*) Scatter plots for the expression of various MHC class I-related genes and the methylation level of the *NLRC5* promoter in melanoma ($n = 468$). (*F*) Scatter plots for *HLA-B* expression and methylation level of *CIITA* promoter in 15 tumor types ($n = 5{,}667$). (*G*) Scatter plots for the expression of *GZMA* or *PRF1* and the methylation level of the *NLRC5* promoter in 15 tumor types ($n = 6{,}528$). (*H*) Scatter plots for *CD8A* expression in 15 tumor types ($n = 6{,}277$) or *CD56* expression and methylation level of the *NLRC5* promoter in 14 tumor types ($n = 5{,}685$). (*I*) Dot plots for the methylation level of various MHC class I-related genes (*x* axis; β values) in all cancer types (16 tumor types, $n = 6{,}557$). The median values are indicated by vertical bars. Statistical significance was determined by the Mann–Whitney test: $^{**}P < 0.01$. (*J*) Spearman rank correlation coefficient between the expression and methylation of indicated MHC class I-related genes in 15 tumor types ($n = 6{,}419$). (*K*) Scatter plots for the expression and methylation level of various MHC class I-related genes in 15 tumor types ($n = 6{,}419$). In *D*–*H*, *J*, and *K* pairwise correlations were calculated using the Spearman's ranked correlation test. *r*, Spearman rho coefficient.

immune evasion among genes involved in the MHC class I pathway. Gene-expression analysis demonstrated that patients with *NLRC5* CN loss showed reduced expression levels of *NLRC5* and of MHC class I and related genes, including *HLA-A*, *HLA-B*, *HLA-C*, *B2M*, *LMP2*, and *LMP7* across the entire cancer cohort (Fig. S3*B*). The various degrees of reduction in *NLRC5* and class I gene expression were observed in samples of numerous cancers that had CN loss, with the highest reduction rate found in breast cancer (Fig. S3 *C* and *D*). To distinguish the effect of CN loss from that of *NLRC5* methylation, cancer groups in which the *NLRC5* promoter is not methylated (β value <0.3) were analyzed for gene expression. Again, patients with *NLRC5* CN loss exhibited decreased expression of *NLRC5* and MHC class I-related genes across the entire cancer cohort and in breast cancer (Fig. 3*C* and Fig. S3*E*), indicating that CN loss of *NLRC5* results in the reduced expression of the genes involved in the MHC class I pathway independently of the methylation level of the *NLRC5* promoter. Collectively, these data indicate that cancer cells selectively lose *NLRC5* at a high frequency, resulting in reduced expression of MHC class I and related genes.

Somatic Mutations in *NLRC5* Are Correlated with Reduced Expression of MHC Class I Genes. Because somatic mutations are another important molecular mechanism of carcinogenesis (30), biopsy samples from 16 solid cancer types were analyzed for somatic mutations in *NLRC5*. A total of 142 patients were found to have mutations, most of which (58.5%) were missense mutations (Fig. 4*A*). Colon cancer patients exhibited the highest *NLRC5* mutation rate (8.6%), followed by melanoma (6.8%) (Fig. 4*B*). Similar to promoter methylation and CN loss, somatic mutations were most frequently observed in *NLRC5* among all MHC class I and related genes (Fig. 4*C*). Mutations were distributed across the entire *NLRC5* coding region with no obvious hot spots (Fig. S4). To determine whether those mutations affect NLRC5 function, mutations ($n = 13$) observed in more than one patient were analyzed for their ability to induce MHC class I gene expression via a reporter gene assay that employs the *HLA-B* promoter and various *NLRC5* expression vectors generated by site-directed mutagenesis (Fig. 4*D*). As shown in Fig. 4*E*, 7 of the 13 *NLRC5* mutants exhibited complete loss of induction for *HLA-B* promoter activity, although it is possible that *NLRC5* mutants that appeared to be functional in this reporter assay may carry altered function at more physiological settings. The data demonstrate that the majority of *NLRC5* mutations in cancer patients are true loss-of-function mutations. Indeed, correlation analysis of *HLA-B* and *NLRC5* expression confirmed the tendency for reduced *HLA-B* expression levels in patients with *NLRC5* mutations compared with patients with wild-type *NLRC5* (Fig. 4*F*). To substantiate this observation further with statistical analysis, we plotted the ratio of MHC class I genes to *NLRC5* to reflect gene induction by NLRC5. As expected, the ratio of MHC class I to *NLRC5* expression was decreased in the *NLRC5* mutant group (Fig. 4*G*). These data indicate that in multiple cancers

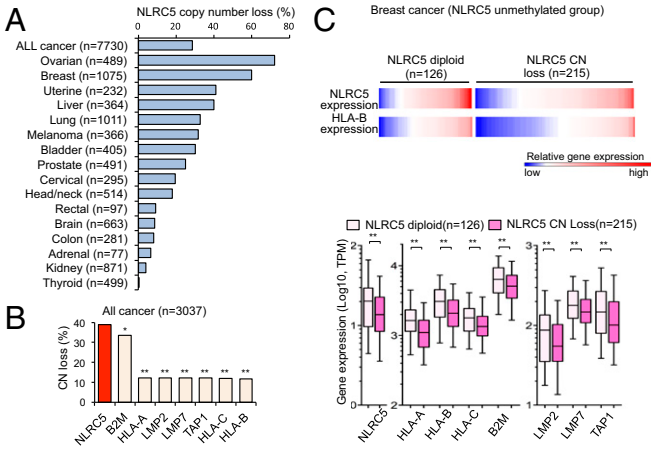

Fig. 3. CN loss in *NLRC5* is associated with reduced expression of MHC class I genes. (*A*) Percentage of cancer patients who carry *NLRC5* CN loss among 16 tumor types. Based on GISTIC values, samples were classified into the *NLRC5* diploid group (GISTIC 0) and the CN-loss group (GISTIC −1 and −2). (*B*) Percentage of cancer patients who carry CN loss of various MHC class I-related genes for nine tumor types for which data are available (bladder, breast, colon, head/neck, lung, ovarian, prostate, rectal, and uterine cancer). Statistical significance was determined by the χ^2 test: $*P < 0.01$; $**P < 0.0001$. (*C*) Heatmap showing gene expression of *NLRC5* and *HLA-B* in the *NLRC5* diploid group ($n = 126$) and in the CN-loss group ($n = 215$) for breast cancer patients in which the *NLRC5* promoter is not methylated (β values <0.3). The box plots show *NLRC5* and MHC class I-related gene expression in the *NLRC5* diploid group or in the CN-loss group in breast cancer. The bar inside the box corresponds to the median, the box corresponds to the 25th–75th percentile, and the error bars indicate the confidence interval (fifth–95th percentile). Statistical significance was determined by the Mann–Whitney test: $**P < 0.01$.

somatic mutations occur preferentially in *NLRC5* as compared with other MHC class I-related genes and are associated with the reduced expression of genes involved in MHC class I-mediated antigen presentation.

The Expression of *NLRC5* Is Correlated with Survival of Cancer Patients. Because MHC class I expression and cytotoxic $CD8^+$ T-cell infiltration in tumors are critical for immunological defense in cancer patients, we analyzed the effect of *NLRC5* on overall survival. Cancer patients were stratified into quartiles based on *NLRC5* expression. The analysis of 5-year survival of patients with 16 different cancer types revealed that the quartile with highest *NLRC5* expression showed significantly better survival than the quartile with lowest *NLRC5* expression in six cancer types (melanoma, rectal cancer, bladder cancer, uterine cancer, cervical cancer, and head/neck cancer) (Fig. 5*A* and Fig. S5*A*). Kaplan–Meier survival analysis also demonstrated that the high *NLRC5* expression was associated with significantly improved cumulative survival in melanoma, bladder cancer, and cervical cancer (Fig. 5*B*). The most striking differences were seen in melanoma and bladder cancer, with 5-year survival rates of 36% and 34%, respectively, in the *NLRC5*-low group compared with 71% and 62%, respectively, in the *NLRC5*-high group. In addition to *NLRC5*, the expression of NLRC5-dependent (*HLA-A*, *-B*, *-C*, *B2M*, *LMP2*, *LMP7*, and *TAP1*) (Fig. 5*C*) but not NLRC5-independent (*Calreticulin*, *Tapasin*, *ERp57*, and *ERAP1*) (Fig. 5*D*) genes involved in MHC class I antigen presentation was positively associated with cumulative survival of melanoma patients. The expression of markers for cytotoxic $CD8^+$ T-cell activity (*CD8A*, *GZMA*, and *PRF1*) (Fig. 5*E*) but not of NK cells (*CD56*) (Fig. S5*B*) also was correlated with better cancer patient survival, most likely through NLRC5-dependent MHC class I antigen presentation. Interestingly, high methylation of *NLRC5* but not of other MHC class I and related genes (*HLA-A*, *-B*, *-C*, *B2M*, *LMP2*, *LMP7*, and *TAP1*) was associated with poor survival in melanoma and bladder cancer, indicating that aberrant epigenetic changes specifically in *NLRC5* in cancer cells impacted clinical outcomes (Fig. 5*F* and Fig. S5 *C* and *D*). Intriguingly, brain cancer (glioma/glioblastoma) showed an opposite correlation, with a significantly lower 5-year survival rate in the cohort with high *NLRC5* expression (Fig. S5*A*). Although the exact mechanism is uncertain, this effect might be caused by the unique anatomy of brain. Because brain mass is limited by the skull, unlike other cancers, one major life-threatening complications of brain tumors is the development of brain edema, which is associated with inflammatory events including impaired blood–brain barrier and destruction of normal brain tissues (31, 32). In fact, patients with brain tumors are commonly treated with anti-inflammatory drugs such as corticosteroids (32, 33). Taken together, these findings show that *NLRC5* expression is correlated with higher survival in multiple cancer types, with the exception of brain cancer, in which it appears to be a negative prognostic factor. Strikingly, DNA methylation of *NLRC5* alone, but not of other MHC class I-related genes, is linked to poor patient survival in melanoma and bladder cancer, further signifying the role of NLRC5 in tumor immunity.

Discussion

This study demonstrates that CITA/NLRC5 is a major target for facilitating immune evasion by cancer cells (Fig. 6). During oncogenic transformation and cancer evolution, tumor cells need to develop ways to escape from the host immune system to sustain development, growth, invasion, and metastasis. Reduction, alteration,

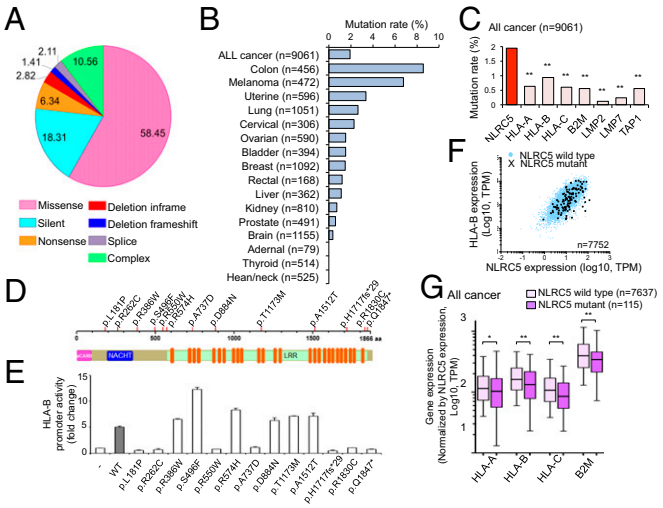

Fig. 4. Somatic mutations in *NLRC5* are correlated with reduced expression of MHC class I genes. (*A*) Pie chart representing the percentage distribution of different types of mutations in *NLRC5* in various cancer patients ($n = 7{,}752$). (*B*) Mutation rate in *NLRC5* for 16 tumor types ($n = 9{,}061$). (*C*) Mutation rate in the indicated genes for 16 tumor types. Statistical significance was determined by the χ^2 test ($n = 9{,}061$): $**P < 0.001$. (*D*) Representation of *NLRC5* indicating 13 mutations found in at least two different cancer patients. (*E*) HEK293T cells were cotransfected with either empty control vector or the respective *NLRC5* mutant plasmid with *HLA-B* reporter plasmid, and *HLA-B* promoter activity was assessed by the dual-luciferase assay and normalized against *Renilla* firefly activity. Data are representative of two independent experiments performed in duplicate and are plotted as fold induction with respect to the control vector. The error bars indicate SD. (*F*) Scatter plots for the expression of *NLRC5* and *HLA-B* for the *NLRC5* wild-type group (blue circle) and the *NLRC5* mutant group (black cross) in 16 tumor types ($n = 7{,}752$). (*G*) Box plots for the expression level of MHC class I-related genes normalized by the expression level of *NLRC5* in 16 tumor types that are either *NLRC5* wild type or *NLRC5* mutant. The bar inside the box corresponds to the median; the box corresponds to the 25th–75th percentile, and the error bars indicate the confidence interval (fifth–95th percentile). Statistical significance was determined by the Mann–Whitney test: $*P < 0.05$; $**P < 0.01$.

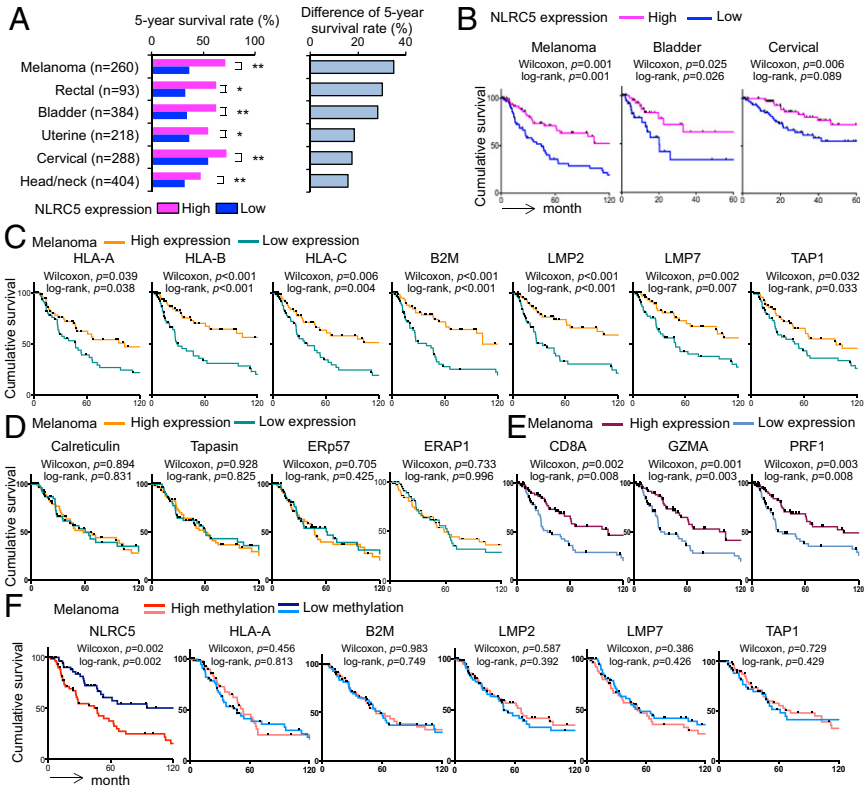

Fig. 5. The expression of *NLRC5* is correlated with better survival in multiple cancer types. Patients were divided into four groups by the level of indicated gene expression or methylation, and the top (high) and the bottom (low) quartiles were analyzed. (*A*, *Left*) Five-year survival rate in high and low *NLRC5* expression groups for indicated tumor types. (*Right*) Difference in the 5-year survival rate in groups with high and low *NLRC5* expression. Statistical significance was determined by the χ^2 test: $^*P < 0.05$; $^{**}P < 0.01$. (*B*) Kaplan–Meier survival curves for indicated tumor types in groups with low and high *NLRC5* expression. (*C*) Kaplan–Meier survival curves for melanoma patients with low and high expression of the indicated NLRC5-dependent MHC class I-related genes. (*D*) Kaplan–Meier survival curves for melanoma patients with low and high expression of the indicated NLRC5-independent MHC class I-related genes. (*E*) Kaplan–Meier survival curves for melanoma patients with low and high expression of *CD8A* and the indicated markers for cytotoxic CD8$^+$ T-cell activity. (*F*) Kaplan–Meier survival curves for melanoma patients with low and high methylation of the *NLRC5* promoter and the indicated MHC class I-related genes. In *B*–*F* statistical significance was determined by the log-rank test and the Gehan–Breslow–Wilcoxon test.

or total loss of tumor antigen expression is critical to avoid killing via activation of cytotoxic CD8$^+$ T cells and can be achieved by at least three mechanisms: (*i*) lack of expression of tumor antigen; (*ii*) loss of MHC class I molecules; (*iii*) impaired function or expression of genes in the class I antigen-presentation pathway such as in the immunoproteasome or class I peptide loading complex in the endoplasmic reticulum (1). Impaired function or expression of CITA/NLRC5, a master regulator of MHC class I genes, affects the latter two steps concurrently (11, 12), thus making NLRC5 an attractive target for cancer cells to evade CD8$^+$ T-cell–dependent immune responses. Indeed, the expression of *NLRC5* is correlated with markers for cytotoxic CD8$^+$ T-cell activity and is associated with better prognosis with prolonged patient survival in multiple cancers (Figs. 1 *E* and *F* and 5 *A* and *B*). Furthermore, the expression of NLRC5-dependent genes (but not the expression of independent genes) involved in MHC class I antigen presentation is associated with cancer patient survival, further supporting the significance of the NLRC5-dependent MHC class I transactivation pathway in antitumor immunity (Fig. 5 *C* and *D*). Several lines of evidence demonstrated that cancer cells have evolved to target *NLRC5* preferentially for immune evasion. First, the *NLRC5* promoter is more highly methylated than any other gene in the MHC class I pathway (Fig. 2*I*). Second, the methylation-mediated suppression of gene expression is most effective for *NLRC5* (Fig. 2 *D*, *J*, and *K* and Fig. S2*I*). Third, among all MHC class I-related genes, CN loss is most frequently observed in *NLRC5* (Fig. 3*B* and Fig. S3*A*). Fourth, somatic mutations were observed more frequently in *NLRC5* than in other MHC class I-related genes (Fig. 4*C*). Strikingly, the methylation status of *NLRC5*, but not of other MHC class I and related genes, was associated with changes in patient survival of melanoma and bladder cancer (Fig. 5*F* and Fig. S5 *C* and *D*). These data identify NLRC5 as a major target of immune evasion in cancers. Although *NLRC5* is expressed in both cancer and infiltrating T cells, it is unlikely that aberrant promoter methylation, CN loss, and mutations in *NLRC5* occur in normal infiltrating cells. Therefore, these data strongly indicate that genetic as well as epigenetic alterations within the cancer cells impact MHC class I-dependent immune responses through altered activity of NLRC5. Although this study focused on the transcriptional regulation of NLRC5, it is possible that NLRC5 may be regulated at the posttranscriptional level, including translational, protein stability, and cellular localization alterations in cancer cells; this possibility needs to be addressed in a future study. Alternative mechanisms by which NLRC5 affects cancer progression could be via regulation of cytokines such as type I IFNs, IL-6, or TNF-α because NLRC5 was reported to be a regulator of TLR response, type I IFN production, and inflammasome activation in early studies. However, it is unclear if these proposed innate immune functions of NLRC5 exist in cancer cells, because

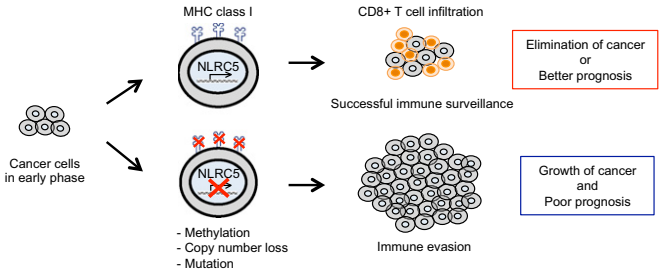

Fig. 6. Model of cancer evolution targeting NLRC5 for immune evasion. NLRC5-dependent MHC class I expression is crucial for CD8$^+$ T-cell–mediated antitumor responses and the elimination of cancer cells. Genetic and epigenetic changes, such as mutation, CN loss, or promoter methylation of *NLRC5* occur during the evolution of cancer cells, leading to an impaired MHC class I system. These changes result in an impaired ability to elicit antitumor CD8$^+$ T-cell responses and reduced infiltration in cancer tissues. Cancer cells successful in immune evasion cause efficient tumor development, leading to poor prognosis of cancer-bearing patients. Cancer cells (gray) and CD8$^+$ T cells (orange) are shown.

the data were not reproducible among different laboratories and by in vivo experiments using *Nlrc5*-deficient mice (12).

Because high expression and low methylation of *NLRC5* are correlated with better survival of cancer patients, these data suggest that *NLRC5* expression and methylation status are useful biomarkers for patient prognosis and survival in multiple cancers. Furthermore, these data indicate that NLRC5 is an attractive therapeutic target in cancer patients. Checkpoint blockade immunotherapy such as anti-CTLA4 or anti–PD-1/PD-L1 therapy has emerged as a leading cancer treatment (34), although its efficacy is hampered when cancer cells successfully evade immune responses. Therapeutics augmenting NLRC5 activity could compensate for this deficit by breaking cancer immune evasion in a broad range of tumor types. Interestingly, it has been reported that currently used therapies, such as an EGF receptor inhibitor (cetuximab) or a B-Raf inhibitor (vemurafenib), enhance MHC class I expression via IFN-γ (35, 36). Therefore, these currently available therapies, originally designed to disrupt oncogenic signaling, may mediate their effects in part via the NLRC5-dependent MHC class I pathway.

Methods

For a more detailed discussion of the materials and methods, see *SI Methods*. Tumor types were selected based on the availability of gene-level RNA-sequencing (RNA-seq) expression data from TCGA. RNA-seq data in normal tissues were from the GTEx web portal. The TCGA abbreviations for the samples used in this study are given in Table S1. The numbers of samples of each tumor type are detailed in Table S2. The primers used for the construction of selected NLRC5 mutant expression vectors are listed in Table S3.

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