T-cell response to p53 tumor-associated antigen in patients with colorectal carcinoma

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Abstract. Despite the radical surgical resection performed in patients with colorectal carcinoma, there is a high rate of tumor recurrence. Over an observation period of 3 years, 18% of the patients in our collective suffered a tumor relapse with local or distinct metastases after initial R0-resection. Some evidence suggests that this may be due to suppression of anti-tumor responses, a phenomenon that might be attributed to regulatory T cells. The aim of our study was to investigate the tumor-specific immune response depending on the UICC stage of patients with colorectal cancer. The cellular immune responses against defined antigens that are overexpressed in most of the patients with colorectal cancer were characterized. For this purpose, the tumor suppressor gene, p53, was chosen as the tumor-associated antigen that exhibits mutations and overexpression in up to 60% of colorectal carcinoma. We observed that p53 induced both IFN-γ and IL-10 secretion. The predominance of IL-10 production indicated that regulatory T cells directly participate in modulating the anti-tumor immune response. IL-10 levels in the blood as well as the expression of regulatory T-cell specific genes at the tumor site correlate with the UICC stage of the disease. These results may provide an explanation for the poor prognosis and increased recurrence rate in patients with advanced carcinoma.

Introduction

Mutations in the p53 gene are the most frequently reported somatic gene alterations in human malignancies. This results in an overexpression and cytosolic accumulation of the p53 protein, a phenomenon observed in more than half of all cancers. This ubiquitous expression of mutated p53 in tumors has prompted immunologists to use p53 as an antigen for the design of immunological therapies in cancer (1,2). The possibility of using wild-type (wt) p53 as an effective tool for immune intervention has been demonstrated in several mouse tumor models. Studies in rodents have demonstrated the immunogenicity of p53 during tumorigenesis and the induction of CD4+ and CD8+ T-cell responses directed to p53 determinants. While T-cell responses to p53 are consistently induced during cancer, they fail to eradicate tumors. It is noteworthy, however, that p53 immunization can lead to the differentiation of p53-specific CD4+ T helper and CD8+ cytotoxic T cells capable of rejecting tumors in mice (3,4). This supports the view that, in the absence of vaccination, anti-p53 T cells capable of rejecting tumors are either never activated or that they are suppressed by other T cells. In support of this view, our laboratory has previously reported that the vast majority of p53-specific CD4+ T cells expanding in mice with J774 sarcomas secrete type 2 cytokines (IL-4, IL-5, IL-10). This observation suggests that in tumor-bearing mice, while p53 is immunogenic it preferentially activates Th2/Treg (Tr1) cells, a response which does not lead to tumor rejection or even protects tumors from eradication by Th1 cells. It is still not clear whether polarization of anti-p53 response to Th2/Treg immunity during tumorigenesis represents a general phenomenon that is relevant to patients with different cancers. As we gain insights into these questions, we may design new methods of therapeutic intervention to manoeuvre the anti-p53 T-cell response towards effective tumor rejection.

Mutations within the p53 gene have been demonstrated in a large proportion of colorectal cancer patients (5). Although MHC class II molecules are usually not expressed on the surface of solid tumors, which makes direct tumor eradication by CD4+ T helper cells impossible, there is cumulative evidence...
of their crucial role in anti-tumor immunity (6). In addition, the recognition by T helper cells of tumor antigens processed and presented by professional antigen presenting cells (APCs), such as dendritic cells (DC), can lead to a variety of events. These events include the activation of anti-tumor specific cytotoxic T lymphocytes (CTL), the production of antibodies by B lymphocytes, and the mobilization of innate effector mechanisms including the activation of tumoricidal macrophages, eosinophils and NK cells (7-10). These results demonstrate that p53-specific T helper cells (Th) can provide help to p53-specific CTL, thereby controlling tumor growth (11,12). Obviously, the p53-specific Th responses detected in patients with primary colorectal cancer fail to ensure tumor eradication, rendering necessary surgical removal of life-threatening tumors. Some so called ‘escape mechanisms’ may account for the lack of tumor eradication by the immune system. They include the loss or downregulation of MHC class I molecules on the tumor surface with a lack of sufficient CTL immunity (13-15), the loss of tumor antigens (16,17), and the lack of co-stimulation followed by T-cell anergy rather than activation (18,19). It is possible that all these events are driven by the production of regulatory cytokines by the tumor by Th2/Treg cells (20,21), but this hypothesis remains to be investigated.

In this study, we evaluated p53-specific T helper immunity in 51 patients with primary colorectal cancer at various UICC stages of disease. We measured p53-specific production to type 1 (IFN-γ) and type 2 (IL-10) cytokine by T cells, the expression levels of p53 in the tumor, the humoral anti-p53 response, and the infiltration of tumor by Th2 cells, which have been associated with a higher level of tumor recurrence and a reduction in disease-free survival (22). We showed that p53 induced both IFN-γ and IL-10 secretion. The predominance of IL-10 production indicated that regulatory T cells directly participate in modulating the anti-tumor immune response. IL-10 levels in the blood as well as the expression of regulatory T-cell specific genes at the tumor site correlate with the UICC stage of the disease.

Patients and methods

Patients. Fifty-one patients diagnosed with primary colorectal cancer were included in this study. The protocol used was approved by the local medical ethics committee. Heparinized peripheral blood and tumor tissue were collected from patients with informed consent before surgery. Blood samples were separated on Lymphoprep according to the manufacturer’s instructions (Nycoderm Pharma, Oslo, Norway). Peripheral blood lymphocytes (PBLs), sera, and tumor specimens were either promptly used or stored at -80˚C. Tumors were evaluated for location, stage, and differentiation grade. Data concerning age, gender, level of wall infiltration, and lymph node metastasis were collected in our database.

Peptides. Ninety-four peptides spanning the whole length of wt p53 protein were synthesized on either polyethylene pins or polystyrene resin following the standard conditions for activation, Fmoc deprotection, and side chain deprotection and cleavage for custom peptides (Mimotopes, Clayton Victoria, Australia). p53 peptides were synthesized as 14-mers overlapping by 4 amino acids and divided into 10 pools: pool 1 (residues 1-50), pool 2 (residues 51-90), pool 3 (residues 91-130), etc.

Humoral response to p53. p53-specific IgG titers were quantified using the commercially available p53 ELISA Kit (Oncogene, Boston, MA, USA). The results for each serum sample were calculated first by determining the relative p53 autoimmune index, following the manufacturer’s calculation protocol (cut off value: 0.035). The anti-p53 IgG titer (U/ml) of the positive serum samples was calculated at 50x dilution using the linear regression curve obtained by plotting the concentration of standards versus the absorbance. In all the ELISA experiments, patient sera were tested in duplicate and the experiments repeated 3 times. Sera from healthy patients were used as controls.

IFN-γ enzyme-linked immunosorbent assay (ELISA). Supernatants from ELISPOT assay (described below) were collected and measured for IFN-γ production by ELISA using BioSource Cytoscreen kits (BioSource, Camarillo, CA), following the manufacturer’s protocol.

ELISPOT assay. The ELISPOT assay used to measure the frequency of p53 reactive T cells producing IL-10 was performed as previously described. Briefly, ELISPOT plates (Millipore, Molsheim, Germany) coated with capture antibody against IL-10 (BD Bioscience, Heidelberg, Germany) in phosphate-buffered saline (PBS) were left overnight at 4˚C. The plates were blocked with 1% BSA in PBS for 2 h and then washed with PBS. PBLs (1x10⁵) in 100 μl of complete RPMI medium (RPMI-1640 medium (Invitrogen, Karlsruhe, Germany) 100 U/ml penicillin (Biochrom, Berlin, Germany), 100 μg/ml streptomycin (Biochrom), 5 mM HEPES (Biochrom), 1% non-essential amino acids (Biochrom) 1 mM sodium pyruvate (Biochrom), and 2x10⁻⁵ M 2-mercaptoethanol (Invitrogen) was added to each well with the addition of the relevant p53 peptide pool (50 μg/ml of each peptide). Control wells contained cells plus either medium alone or PHA (final concentration, 5 μg/ml) without the peptide. After 24 h, the plates were washed and a biotinylated detection antibody (BD Bioscience) was added for 2 h at room temperature. After further washing, horseradish peroxidase (HRP) conjugate (BD Bioscience) was added for 1 h at room temperature in darkness. The development was performed with freshly prepared aminoethyl carbazole (AEC) (Pierre pharmaceuticals, Rockland, IL; 10 mg/ml in N, N-dimethylformamide) in 0.1 M sodium acetate buffer (pH 5.0) mixed with 30% H₂O₂ (200 μl/well). The resulting spots were counted on a computer-assisted ELISA Spot Image Analyzer (CTL, Cleveland, OH). All experiments were repeated four times and the results are expressed as the frequency of cytokine-producing cells per million cells.

Immunohistochemistry. Representative biopsies of tumor samples were fixed in 10% buffered formalin for histological examination and PCR-analysis. Paraffin sections were evaluated using hematoxylin and eosin, and periodic acid Schiff (PAS). Additional biopsies were snap-frozen and stored at...
-80°C for RNA extraction and RT-PCR analysis. Cryostat sections fixed in paraformaldehyde-lysine-periodate were prepared for cell-surface antigen staining. Monoclonal antibody (mAb), DO-7 (anti p53, 35-45), from DakoCytomation (Heidelberg, Germany); anti-CD4-mAb, anti-CD25-mAb, control mAbs and secondary antibodies from BD Pharmingen (Heidelberg); and Isotype-matched mAbs or purified IgG1 and controls for residual endogenous peroxidase activity were included in each experiment. The percentage of tumor cell staining for p53 and the intensity of staining of the nucleus (low, medium, or high) was estimated within 20 consecutive high-power fields (magnification x40).

**RNA extraction.** RNA was extracted using an RNA extraction kit (Qiagen, Hilden, Germany) from at least 10 mg of homogenized tumor tissue, washed in DEPC-75% ethanol before being dissolved in DEPC-water, and stored at -70°C until further analyses. The amount of total RNA was determined by measuring absorbance at 260 nm. The purity of the total RNA was established by confirming that the 260 nm:280 nm ratio was within a 1.8-2.0 range, indicating that the RNA preparations were free of protein contaminants.

**Reserve transcriptase polymerase chain reaction (RT-PCR) for detection of p53 mutations.** cDNA was prepared from 2 μg of total RNA using the Promega AccessQuick™ RT-PCR System (Promega, WI, USA). The procedure was performed according to the standard RT-PCR protocol using AMV-reverse transcriptase. Two sets of primers amplifying the p53 coding region spanning exons 4 and 9 were used. The primer sequences were as follows: for exons 4-6, sense primer TGT CCC CGG ACG ATA TTA AAC and antisense primer TTC CTT CCA CTC GGA TAA GAT GC (amplicon size 465 bp); and for exons 5-9, sense primer GCT CAG ATA GCG ATG GTC TGG C and antisense primer TCT CGG AAC ATC TTC TCG AAG CG (amplicon size 484 bp). RT-PCR conditions for the first reaction were 48°C for 45 min, followed by 2 min at 95°C, followed by 40 cycles of 94°C for 30 sec, 60°C for 60 sec, and 68°C for 20 sec; followed by 68°C for 5 min. Before sequencing, all PCR products were purified following the QiAquick PCR purification kit protocol (Qiagen) using vacuum manifold. PCR products were blindly sequenced on an ABI PRISM 373 fluorescent dye terminator (PE/ Applied Biosystem, Foster city, CA, USA).

**Real-time polymerase chain reaction (real-time PCR) for Th2 specific genes in tumor specimens.** mRNA expression of representative surface molecules and cytokines (CD4, CD25, Foxp3, GATA-3, CTLA-4, GITR, IL-10, IFN-II) was analyzed by using 2 μg of heat-denatured RNA. The following primer sequences were used (Sigma Genosys, Woodlands, TX): for GAPDH, 5'-ATC GCC CTA TCA TGG GCC AAG GTG TGC TGG GC-3' and 5'-ACC ACC TGT GCA ACG CCT GC-3'; and for GATA-3, 5'-GGT CAG TGC CAT TTT CCC AG-3'; for Foxp3, 5'-GAGAAGCTGAGTGCCATGCA-3'; for CTLA-4, 5'-TCC CTG CAG TGA CCT GGA TGT CCA GGT GC-3'; for CD25, 5'-AAG TCC AAT GCA GTG TCT CAG CTG GAG C-3' and 5'-GAC AGT GCA GAPDH, 5'-ATC CCA TCA CCA TCT TCC AGG-3' and 5'-CAC GGC CTT GCT CTT GTT TT-3'; and for IFN-II, 5'-TGT TGA ACT GCA CTG GCC A-3' and 5'-CAC CCA GGG CAG CTG CT-3'. Primers were designed using the Primer Express software for primer design to amplify short segments of 50-150 base pairs of target cDNA. Optimum primer concentration was determined by titration. Real-time quantitative PCR was performed in a two-step RT-PCR using SYBR-Green PCR Master Mix (PE Biosystems, Foster City, CA) with 100 ng cDNA and 300 nM of primers in a total reaction volume of 50 μl. PCR thermal cycling conditions were as follows: 95°C for 10 min, followed by 40 cycles of 95°C for 15 sec and 60°C for 60 sec. Gene specific products were continuously measured by an ABI PRISM 7700 sequence detector (Applied Biosystems, Foster City, CA) and relative quantification was performed following the manufacturer's instructions. All samples were assayed in duplicate and normalized during data analysis by a passive reference dye provided in the SYBR-Green PCR Master Mix to compensate for well-to-well fluorescence variations. The average threshold cycle (Ct) value was calculated as the cycle number at which the fluorescence of the reporter reaches a fixed threshold. The difference (∆Ct) between the average Ct values of the samples in the target wells and those of the housekeeping gene, GAPDH, was assessed, followed by the calculation of the difference between the average ∆Ct values of the samples for each target and the ∆Ct value of the control sample for that target (∆∆Ct). The relative quantification value, fold change, is expressed as 2-∆∆Ct.

**Statistical analysis.** To compare the significance in differences in non-related data, the Mann-Whitney U test was performed. Significance was assumed if p<0.05.

**Results**

**Humoral response to p53.** We studied the relationship between the p53-specific antibody response and the expression of p53 in the tumor. The results were correlated with the stages of disease (UICC stage I-IV). All 51 patients, including the 20 for which PBLs were collected, were tested for the presence of p53-specific IgG serum antibodies and 39.2% of the patients displayed p53-specific IgG serum antibodies (n=20). Antibodies were not detectable or were below the critical range in 31 patients, these results were not taken into consideration. 25% of the patients with a positive antibody status were UICC stage I, 20% were UICC II, 20% were UICC III, and 35% were UICC IV. The majority of all tested patients (74%) showed a negative serum response to p53 IgG. No correlation was found between the levels of free p53 protein and the antibody levels of the patients (data not shown). Immunohistochemical analysis of p53 expression in the tumors showed that tumors of higher stages had a significantly stronger staining intensity than tumors of lower UICC stages (UICC III and IV 57.1% vs. I and II 37.1% positive cells expressing...
p53-specific IgG antibodies correlated with a strong p53 staining intensity (p<0.05), suggesting a correlation between the intracellular accumulation of p53 in the tumor cells and the humoral response to p53 in accordance with previous studies (23,24). In contrast, both the presence of a humoral response and UICC stage of disease showed no correlation with levels of free p53 protein in the sera.

**p53-specific T helper cell reactivity.** Tumor-specific immune responses in patients with colorectal cancer in different UICC stages of disease were analyzed and their wild-type p53-specific regulatory T-cell function was characterized. After stimulation with the p53 peptide pools, two peptide pools (AA 1-50 and AA 291-330) were found to induce IL-10 production. We observed that the UICC stage was important in IL-10 production in response to the p53 peptide, AA 1-50. More Th2 cells producing higher IL-10 levels in response to the wild-type p53 peptide sequence, AA 1-50, were seen in patients with UICC stages I and II (n=9) than in those with stages III (n=8) and IV (n=8) (mean value, I/II: 135±16 spots/10⁵ cells, vs. III: 118±20 spots/10⁵ cells, vs. IV: 90±17 spots/10⁵ cells, p<0.05). A decreased IL-10 expression was observed in the presence of the peptide pool, AA 291-330, in PBLs from patients in UICC stage III (mean value, I/II: 58±17 spots/10⁵ cells, vs. III: 26±12 spots/10⁵ cells, vs. IV: 62±13 spots/10⁵ cells, p<0.05) (Fig. 2). In contrast, p53 peptide sequence AA331-370 caused IFN-γ production and no correlation was found between the UICC stage and the intensity of the Th1 immune response (Fig. 3). The presence of T helper immunity is not necessarily linked with a p53-specific IgG response. Pools of ten peptides were used in this study. More determinants inducing a Th2 type immune response than Th1 type were observed along the whole p53 protein sequence: AA 1-50 and AA 291-330 inducing IL-10 production vs. AA 331-370 inducing IFN-γ production, indicating that the specific immune responses to p53 depend on the presented p53 protein determinant.

**p53-specific humoral and cellular immunity in relation to p53 mutational status.** p53 mutational status was analyzed in 16 of these patients with colorectal carcinoma. Five different point mutations located at 5 different sites along the p53 sequence were identified in their solid tumor biopsies [base pair (bp) 82 C:G, bp 300 T:C, bp 366 A:G, bp 686 T:C, bp 711 T:C]. p53 mutations showed no significant correlation with either the presence of p53-specific antibodies or elevated T-cell reactivity (data not shown). This observation is in concordance with previous studies demonstrating wild-type p53-specific T helper cell responses in patients with colorectal cancer, indicating that neither the presence of p53-specific antibodies nor the T helper immune response is necessarily linked to mutations in the p53 gene (25).

**Characterization of tumor infiltrating lymphocytes by analysis of Th2 specific gene expression.** Real-time PCR analysis revealed an increased expression of T regulatory specific genes (CD4, CD25, CTLA4, Foxp3, GATA-3, GITR, IL-10) at the tumor site in patients with UICC stage II (CD4, 13.3±5 ΔCt; CD25, 11.3±3 ΔCt; CTLA4, 20±7.1 ΔCt; FOXP3, 23.8±9.3 ΔCt; GATA-3, 21.8±7.1 ΔCt; GITR, 20.4±6.4 ΔCt; IL-10, 10.1±4.9 ΔCt). This indicates that, at this stage of disease, the population of regulatory tumor-specific T cells indeed comprises of CD4⁺CD25⁺ cells (Fig. 4).
Effector cells (CD8, IFN-\(\gamma\)) and apoptotic (Fas/FasL) specific genes were also elevated in these patients at stage II (CD8, 8.6±3.2 \(\Delta Ct\); IFN-\(\gamma\), 16.3±5.3 \(\Delta Ct\); Fas, 31.2±7.7 \(\Delta Ct\); FasL, 18.3±5.1 \(\Delta Ct\)), suggesting that events important for the fate of the immunological response appear in an early stage of cancer development. The genes for T regulatory cells (CD4, CD25, CTLA4, Foxp3, GATA-3, GITR, IL-10) and for effector cells (CD8, IFN-\(\gamma\), Fas/FasL) were significantly decreased in UICC stages III and IV compared to stage II (p<0.05).

Remarkably, in our immunohistochemical staining experiments, higher numbers of CD4+CD25+ T lymphocytes were detected at the tumor site of patients with UICC stage II than in patients of later disease stages (Fig. 5, CD4+CD25+ in UICC II vs. CD4+CD25+ in UICC IV, p<0.05).

Discussion

A number of observations show that CD4+ T-cell responses directed to p53 are elicited during the process of tumorigenesis in vivo. First, anti-p53 antibodies displaying IgG isotypes have been detected in the blood of cancer patients (26-30). Since B-cell activation and differentiation are known to require help from activated Ag-specific CD4+ T cells, it is
likely that some anti-p53 CD4+ Th cells are stimulated during tumor development. Second, in patients with breast tumors expressing mut p53, PBMC have been shown to proliferate in vitro in the presence of p53 protein (28). While these studies suggest that anti-p53 CD4+ T cells become activated during cancer, the nature of the p53 determinants recognized
by these CD4+ T cells as well as the functional properties of these T cells are still unknown. It has been demonstrated that anti-p53 T cells specific to certain wild-type p53 determinants are present in the periphery of the adult immune system and can be specifically activated after p53 peptide immunization in tumor-bearing mice (31). More importantly, mice inoculated with syngeneic J774 metastatic sarcomas mount a potent CD4+ T-cell response to p53. This response is mediated by T cells recognizing the mutated portion of p53 and by T cells directed to formerly cryptic self-p53 determinants. Interestingly, the anti-p53 Th response directed toward distinct p53 peptides depends upon the stage of tumorogenesis (31). In the present study, we demonstrated that the presence of p53-specific IgG antibodies correlates with the strong p53 staining intensity in the tumors of patients with primary colorectal cancer, although just a minority of the examined patients (39%) displayed a p53-specific humoral response. This correlation between intracellular accumulation of p53 in the tumor cells and the presence of humoral immunity to p53 is in accordance with previous studies (32).

Cytokine production analysis shows the UICC stage-dependent wild-type p53-specific Th2 immune response in these patients, which is not necessarily associated with the presence of p53-specific IgG responses or specific mutations in the p53 gene. Upon stimulation of lymphocytes derived from patients with wild-type p53 peptide pools, distinct residues induced a Th2 (IL-10) or Th1 (IFN-\(\gamma\)) type response. T cells from the patients in UICC III expressed higher IL-10 levels in response to p53 peptide AA 291-330 than patients in UICC III or IV, indicating that T cells display a UICC stage-dependent IL-10 production in response to p53 peptides. In contrast, other p53 peptides (AA 331-370) led to IFN-\(\gamma\) production but no correlation was observed between the UICC stage and the Th1 response in the ELISPOT analysis. This is consistent with our previous study in leukemic mice showing that T cells respond to different p53 peptides depending upon the stage of metastasis. Interestingly, elevated numbers of CD4+ CD25+ lymphocytes in the tumor infiltrating fraction were observed in patients with early-stage carcinoma. These results suggest that the type of tumor-specific immune response to p53 at early stages of colorectal cancer depends on the presentation and recognition of specific wild-type p53 residues. It is noteworthy that the majority of p53 determinants that are immunogenic in colorectal cancer patients induce the activation of IL-10-producing T cells. This may explain the dominance of the type 2 T-cell response and concomitant lack of effective tumor rejection in these patients.

Both the p53-specific cellular and humoral reactivity showed no significant association with mutations within the p53 gene. These findings are in accordance with previous studies demonstrating the absence of correlation between p53-specific T-cell immune response in colorectal cancer patients and p53 gene mutations (33). This may suggest the presence of T-cell responses to conserved regions of p53 antigen.

Our study suggests that, through the overexpression of p53, the tumor may directly influence the outcome of immunological surveillance in patients with primary colorectal cancer in the early course of cancer development. This overexpression of p53 may lead to the induction of p53-specific Th2 cells and, subsequently, to the suppression of the Th1 type immune response by immunosuppressive cytokines such as IL-10. Multiple studies in transplantation immunology have demonstrated that Th2 type immune responses induce tolerance rather than rejection of ‘foreign’ tissue (34). Thus, considering a tumor a ‘modified self-tissue’ similar to a transplanted organ, the overexpression of p53 in the tumor could facilitate the inhibition of anti-tumor immune activity via Th2 cell stimulation (35,36). This is likely to represent a mechanism by which a tumor escapes immune surveillance. This may explain why p53 overexpression is more frequently found in many malignancies with a poor prognosis (37,38). The observation that the immunological response exerted by regulatory and effector T cells is downregulated in UICC stages III and IV indicates that the events important for the fate of the immunological response appear in the early stages of colorectal cancer development.

Two additional mechanisms related to the presentation of p53 peptides to Th2 cells could also play a role in the Th1/Th2 imbalance detected in patients with colorectal cancer. First, the failure to induce immediate effector functions mediated by CTLs could be due to insufficient amounts of p53 determinants. Our results and other studies show that colorectal tumors of early stages may be too small to release enough p53 peptides for efficient sampling by antigen presenting cells resulting in the predominance of the Th2 arm of the anti-tumor immune response (39). Second, the intestinal microenvironment which is of an anti-inflammatory rather than a pro-inflammatory nature inhibits a sufficient delivery of pro-inflammatory signals to DCs. In this case, DCs would stay in an immature state of low antigen capture activity and secretion of IL-12 (40). This would lead to a failure of polarization toward a Th1 immune response in the draining lymphoid nodes of a colorectal tumor even if sufficient amounts of p53 epitopes are presented (41). More experiments are required to verify this hypothesis. Nevertheless, our study offers a rational explanation for the failure of some clinical vaccination trials which have been undertaken with the intention to treat patients with colorectal cancer. It might be that the fate of the immunological response is already determined in early cancer stages and, therefore, these clinical trials are initiated too late in the course of tumor progression.

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