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SSAT PLENARY PRESENTATION

Novel Vaccine Targeting Colonic Adenoma: a Pre-clinical Model

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Abstract

Background Colorectal cancer (CRC) is the second leading cause of cancer-related mortality in the USA. Over 80% of CRC develop from adenomatous polyps. Hence, early treatment and prevention of adenomas would lead to a significant decrease of disease burden for CRC. MYB is a transcription factor that is overexpressed in both precancerous adenomatous polyps and colorectal cancer, and hence an ideal immunotherapeutic target. We have developed a cancer vaccine, TetMYB, that targets MYB and aim to evaluate its efficacy in the prophylactic and therapeutic management of adenomatous polyps.

Material and Methods Six- to eight-week-old $Apc^{min/+}$ (Familial Adenomatous Polyposis model) and Apc580S (sporadic model) C57BL/6 mice were used. The $Apc^{min/+}$ mice are carried a germline mutation of one Apc allele whereas the Apc580S model has an inducible silencing of one Apc allele, when exposed to tamoxifen, via the Cre-Lox recombination enzyme system. In the prophylactic treatment group, $Apc^{min/+}$ and Apc580S C57BL/6 mice were vaccinated and surveyed for clinical signs of distress. Number of adenoma and survival were measured. In the therapeutic cohort, Apc580S C57BL/6 mice were given tamoxifen-laced food to activate Cre-Lox recombinase mediated silencing of one Apc allele and thus inducing adenoma development. Following adenoma detection, mice were vaccinated with TetMYB and treated with anti-PD-1 antibody and were analyzed for overall survival.

Results In both the prophylactic and therapeutic setting, mice vaccinated with TetMYB had a significantly improved outcome, with the vaccinated $Apc^{min/+}$ mice having a median survival benefit of 70 days ($p = 0.008$) and the vaccinated Apc580S mice having a mean survival benefit of 134 days ($p = 0.01$) over the unvaccinated mice. In the prophylactic cohort, immunofluorescence confirmed a stronger cytotoxic $CD8^+$ T cell infiltrate in the vaccinated group, implying an anti-tumor immune response. In the therapeutic cohort, vaccinated Apc580S mice showed significantly reduced adenoma progression rate compared to the unvaccinated mice ($p = 0.0005$).

Conclusion TetMYB vaccine has shown benefit in a prophylactic and therapeutic setting in the management of colonic adenoma in a murine model. This will form the basis for a future clinical trial to prevent and treat colonic adenomatous polyps.

Keywords Colorectal adenoma · Colorectal cancer · Immunotherapy · Vaccination · Checkpoint inhibition blockade · Mouse model

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Background

Colorectal Adenoma and Cancer Risk

Colorectal cancer (CRC) is the third most common cancer and the second leading cause of cancer-related mortality in the USA.[1] Most colon cancers (> 80%) develop from precursor adenomatous lesions through the "classical" chromosome instability pathway.[2,3]

The development of adenoma and progression to cancer is accelerated in the familial adenomatous polyposis syndrome (FAP) population due to a germline *APC* gene mutation. With respect to colorectal cancer risk, these patients rarely develop cancer before age 20[4] but there is debate regarding the timing of definitive surgery with a balance between cancer risk and family planning implications.[5]

Therefore, new and less invasive treatment options are urgently needed for these at-risk populations.

Colorectal Cancer Immunotherapy

Accumulated evidence has shown that a poor immune response against CRC, as defined by the absence of tumor-infiltrating lymphocytes (TILs), particularly $CD8^+$ T cells, within the primary tumor is a strong predictor of cancer relapse.[6,7] This has led to the concept that a specific reawakening of the latent immune response may provide the basis for therapeutic strategies that are aimed at improving CRC tumor control.

In this context, immune checkpoint inhibitors that target the programmed death receptor, PD-1, and its ligand, PD-L1, have become exciting and effective clinical targets for the treatment of CRC. A landmark publication demonstrated the potency (~ 70% response rate) of immune checkpoint blockade in microsatellite unstable/high (MSI-H) CRC therapy using anti-PD-1 antibodies.[8]

MSI-H CRCs typically have high TILs that we now understand have the capacity to respond to PD-1/PD-L1 therapy and clear tumors. The basis for this high TIL count is presumed to be due to increased "neo-antigen" expression by the MSI-H CRC that is recognized by the TILs. However, in the majority of CRC (> 85%) that are microsatellite stable (MSS), no benefit was recorded with anti-PD-1 therapy.[8] This observation coupled with the finding that the absence of TILs in MSS CRC predicts poor outcome has led to the growing view that the immune response against CRC is central to determining patient outcome. Indeed, this view led to the development of the *Immunoscore* by Galon and others.[9,10]

Cancer vaccine is one of the available therapeutic options for low antigenic cancers, such as MSS CRC, to prime the immune system to recognize neo-antigens or overexpressed self-antigens of the tumor.[11] Thus far, most cancer vaccines have demonstrated minimal clinical benefit due to immune suppressive mechanisms employed by tumors; one such example is a peptide vaccine against MUC1 TAA whose efficacy was hampered by myeloid-derived suppressor cells (MDSCs).[12] The recent introduction of checkpoint inhibitor blockade as an adjunct to cancer vaccines has shown promising results.[13]

Myb Oncoprotein and TetMYB Vaccine

Our laboratory (Ramsay Laboratory, Peter MacCallum Cancer Center, Australia) has developed a novel DNA cancer vaccine, TetMYB, which targets the Myb oncoprotein and has shown partial efficacy in two pre-clinical models of CRC.[14] Myb is a DNA-binding transcription factor that plays an important role in cellular proliferation and differentiation.[15] In malignancies of epithelial nature such as CRC, *MYB* is aberrantly expressed and when expressed at the high range, it correlates with poor prognosis for patients with CRC.[16]

More importantly and pivotal to our proposed CRC prevention therapy study, *MYB* expression is also more pronounced in colon adenomas.[17] In this current study, we investigate the efficacy of TetMYB against adenoma in a prophylactic and therapeutic settings in pre-clinical models of sporadic adenoma and FAP.[18]

Materials and Methods

Animal Models

To mimic the initiating event of the majority of CRC (i.e. loss of heterozygosity of the *APC* gene), the transgenic $Apc^{min/+}$ and Apc580S mouse models were selected. The $Apc^{min/+}$ model carries a heterozygous germline *Apc* mutation and spontaneously forms gastrointestinal tract adenomas, in addition to extra-intestinal manifestations, and thus represents FAP. The Apc580S model carries a tamoxifen-activatable (ERT2) intestinal–specific Villin-Cre (VilCre) recombinase system on one *Apc* allele, which allows for conditional silencing of one *Apc* allele and inducing adenoma formation, thus representing sporadic adenoma.

Six- to eight-week-old $Apc^{min/+}$ and Apc580S mice were bred in the Peter MacCallum Cancer Center (Melbourne, Australia) animal facility. $Apc580S^{lox/lox}$ mice were crossed with VilCre(ERT2) Rosa26 mice to generate an $Apc580S^{lox/+}$ VilCre(ERT2) Rosa26 mice (referred to as Apc580S). All mice were of a C57BL/6 background. All care and use of animals were conducted in compliance with the Australian Code of Practice for the Care and Use of Animals for Scientific Purpose. Ethical approval for this project was obtained from the Peter MacCallum Cancer Center Animal Experimentation Ethics Committee.

TetMYB Construct

The construction of the TetMYB vaccine was previously published by our laboratory.[14,19] The P2-*Myb*-P30 reading frame was constructed by ligating DNA sequences encoding immunodominant MHC class II-restricted tetanus peptides, P2 and P30, to the amino and carboxyl termini of a full-length mouse *Myb* cDNA transgene in which inactivating mutations had been introduced.[14,20] The use of the full-length c-Myb cDNA as tumor antigen allows for whole c-Myb antigen presentation to the immune system in the form of processed peptides. This is because all cellular proteins are cleaved by the proteasome and presented as 8–10 amino acid peptides on the MHC class I. The P2 and P30 tetanus domains are used to break peripheral tolerance by associative recognition, a process whereby a dominant epitope on a fusion protein enhances the response to other epitopes.[21] To isolate and confirm the immunological effects that we observed were Myb specific

rather than effects of the Tet domains, we replaced the *Myb* sequences with those encoding for green fluorescent protein (*GFP*) resulting in TetGFP. TetGFP is not intended to assess transfection, translation, or expression of the vaccine.

TetMYB Manufacture

The manufacture of the TetMYB vaccine plasmid consists of two parts. Firstly, a molecular cloning approach that utilizes Gateway recombination technology (Invitrogen, Carlsbad, CA) to build the TetMYB vaccine plasmid. This involves coupling tetanus domains to both ends of a full-length Myb gene, where three inactivating mutations were inserted to negate its function as an oncogene. This P2-*Myb*-P30 fusion gene is then inserted into the pVAX1™ plasmid (Thermo Fisher Scientific, Waltham, MA). This plasmid was then transfected into One Shot™ MAX Efficiency™ DH5α™-T1R Competent Cells (Thermo Fisher Scientific, Waltham, MA) to yield a clonal population of transformed cells.

The second part involves amplification of these transformed cells using bacterial cultures and extraction and purification using the Qiagen™ (Hilden, Germany) plasmid purification kit to yield sufficient quantities of TetMYB for these experiments.

Experimental Design

Prophylactic Vaccination of $Apc^{min/+}$ and Apc580S Mice

Eight-week-old $Apc^{min/+}$ mice were allocated into three groups: (i) unvaccinated and vaccinated with either (ii) TetGFP (15 μg/50 μL dH_2O), or (iii) TetMYB (15 μg/50 μL dH_2O) intramuscularly for a total of three fortnightly vaccinations. Endpoints were > 20% weight loss, blood-stained feces, or other accepted ethical endpoints.

Six-week-old Apc580S mice were allocated into two groups: (i) unvaccinated and (ii) TetMYB intramuscular vaccination for a total of 3 fortnightly doses. Having shown that the TetGFP control vaccine afforded no significant protection against unvaccinated group, we then proceeded to additional studies omitting this treatment to minimize the unnecessary use of mice. The intestinal-specific VilCre recombinase was induced at week 12 by replacing food pellets for 24 hours with tamoxifen-laced food. This consisted of 0.1 g tamoxifen citrate (Sigma Aldrich, St. Louis, MO, USA) in 2 mL absolute ethanol, 120 g Ensure (Abbott Nutrition, Columbus, OH, USA), and 12 g sucrose in 100 mL drinking water. Endpoints were > 20% weight loss, blood-stained feces, or other accepted ethical endpoints.

At endpoint, mice from both groups were euthanized for tissue collection. Investigators were blinded to treatment group allocation.

Therapeutic Vaccination of Apc580S Mice

Six-week-old Apc580S mice were individually caged, fasted for 24 hours, and induced with tamoxifen-laced food for 24 hours. Adenoma formation was monitored by weekly colonoscopy commencing at 4 weeks following induction based on published data.[18] Upon detection of adenoma, mice were treated weekly with 4 doses of TetMYB (intradermal 15 μg/50 μL dH_2O with 5% DMSO) + anti-PD-1 (intraperitoneal RMP1-14 200 μg/100 μL PBS) followed by 2 doses of TetMYB alone.

Evaluation of Adenoma Growth Using Colonoscopy

Colonoscopy was performed to monitor the development and progression of adenoma. Equipment used were 2 mm 0° mouse colonoscope, laparoscopic camera, fiber optic light cable and insufflation sheath, all attached to an endoscopy stack consisting of video processor, light source and insufflator (Karl Storz GmbH, Tuttlingen, Germany). Direct video feed to perform the procedure was displayed on a laptop computer using an external video capture card and software (BlazeVideo, Shenzhen, China), which also allow capture of photos for analysis. Mice undergoing the procedure were anesthetized using an oxygen-isoflurane mixture delivered via a nose cone according to institutional protocols. Treatment response was monitored and quantified using a ratio of adenoma-area to lumen-area ratio, which corrects for parallax error (Fig. 1a). The endpoint is complete luminal obstruction by adenoma as demonstrated on colonoscopy (Fig. 1b). Colonoscopy and photography were performed by two investigators. Photographs were then de-identified and analysis was performed by a separate investigator who was blinded to treatment allocation.

Histology and Immunohistochemical Analysis

Harvested small intestine and colon were either formalin-fixed paraffin-embedded for immunohistochemistry or frozen in OCT™ compound (Sakura, Torrance, CA, USA) for immunofluorescence. For immunofluorescence detection of $CD8^+$ T cells, frozen tissue was sectioned (4 μm) and stained with $CD8^+$ and isotype control antibodies (Becton Dickinson Biosciences, New Jersey, USA). In brief, frozen tissue sections were fixed in cold acetone for 10 minutes. The slides were incubated with primary antibody for 90 minutes and secondary antibody for 75 minutes at room temperature. ProLong® Gold mounting reagent with DAPI (Invitrogen, Carlsbad, CA, USA) was added to the section and slides were cover slipped.

For immunohistochemistry, paraffin-embedded sections were de-paraffinized with xylene and rehydrated. Antigen retrieval was performed in 1 mM EDTA buffer in a pressure cooker. Sections were then treated with hydrogen peroxide and incubated with primary antibody (c-Myb and β-catenin)

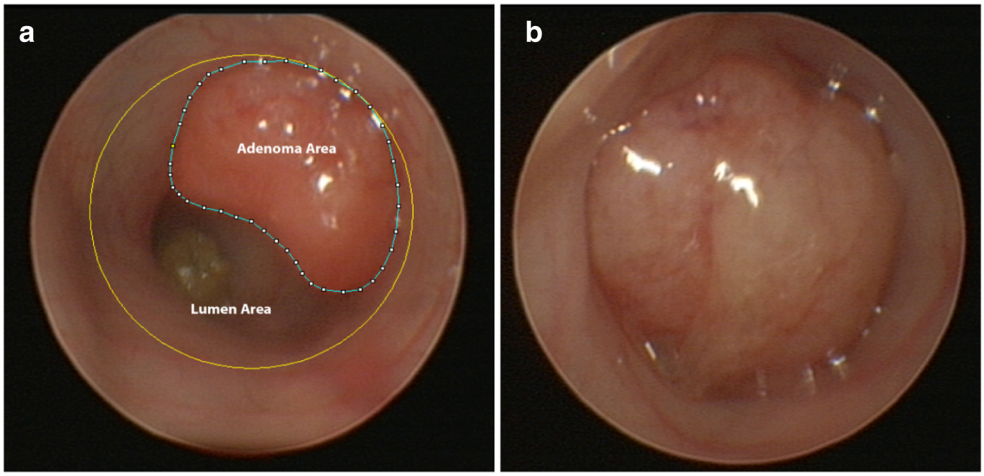

Fig. 1 Measuring adenoma area to lumen area ratio (**a**). Endpoint as complete obstruction by adenoma as seen on colonoscopy (**b**)

overnight at 4 °C. HRP-tagged secondary antibody was added for 30 minutes at room temperature and Dako DAB substrate (Agilent Technologies, Santa Clara, USA) was used for visualization. All microscopic images were taken using Olympus BX-51 microscope (Olympus Corp., Tokyo, Japan).

Gene Expression Analysis

RNA was extracted from the colon of wild-type C57BL/6 and colonic adenomas of $Apc^{min/+}$ and Apc580S mice using RNA extraction kit (Qiagen, Hilden, Germany). RNA was transcribed into cDNA and analyzed for *c-Myb* expression using Fast SYBR Green Mix (Applied Biosystems, Foster City, USA). *c-Myb* expression was normalized against the housekeeping gene *β-2 microglobulin* (*β-2m*).

Statistical Analysis

Data was analyzed using GraphPad Prism Version 7 for Mac, GraphPad Software, La Jolla, CA, USA, www.graphpad.com. The statistical methods used are mentioned in the figure legend.

Results

$APC^{min/+}$ Mouse Model

TetMYB Confers Significant Survival Advantage as Prophylaxis

The $APC^{min/+}$ mice present a pre-clinical model for FAP, as mice spontaneously form gastrointestinal tract adenomas.[22] In our study, mice were allocated into the following groups: unvaccinated ($n = 7$), TetGFP ($n = 10$), and TetMYB vaccine ($n = 8$). Their mean survival following vaccination on week 8 were 141 (± 40 days SD), 170 (± 31 days SD), and 211 days (± 45 days SD), respectively (Fig. 2). There was a significant survival benefit of the TetMYB-treated group compared to the unvaccinated group ($p = 0.008$) and TetGFP-treated group ($p < 0.04$), but no difference between TetGFP-treated and unvaccinated controls ($p = 0.11$). *c-Myb* overexpression in $Apc^{min/+}$ adenomas ($p = 0.037$) was confirmed by qRT-PCR for *c-Myb* mRNA expression by comparing adenomas harvested from $Apc^{min/+}$ mice and wild-type C57BL/6 intestinal crypts (Fig. 3a). Furthermore, adenomas of all groups were analyzed for their cytotoxic ($CD8^+$) T cell infiltration. Adenomas harvested from TetMYB vaccinated animals showed a significant increase in their cytotoxic T cell infiltrate compared to the TetGFP treated group ($p = 0.02$) (Fig. 3b–d).

Apc580S Mouse Model

TetMYB Confers Significant Survival Advantage as a Prophylaxis

The Apc580S mouse model is a tamoxifen-inducible Cre-mediated pre-clinical model for sporadic adenoma formation. Apc580S mice were vaccinated 6, 8, and 10 weeks after birth and received tamoxifen-laced food in week 12. When examining prophylactic vaccination of Apc580S mice, the TetMYB

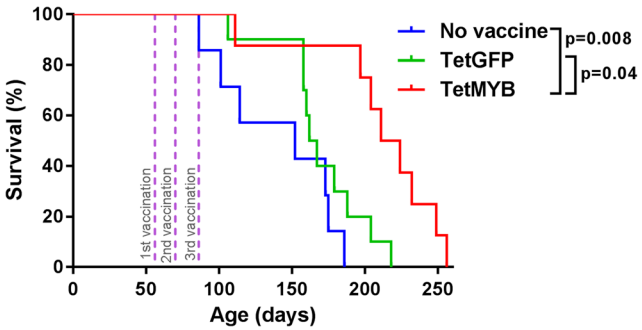

Fig. 2 Prophylactic TetMYB vaccination resulted in significantly longer survival over both TetGFP and unvaccinated $Apc^{min/+}$ mice (log-rank (Mantel-Cox) test). TetGFP was used as control for effect of Tet domains alone

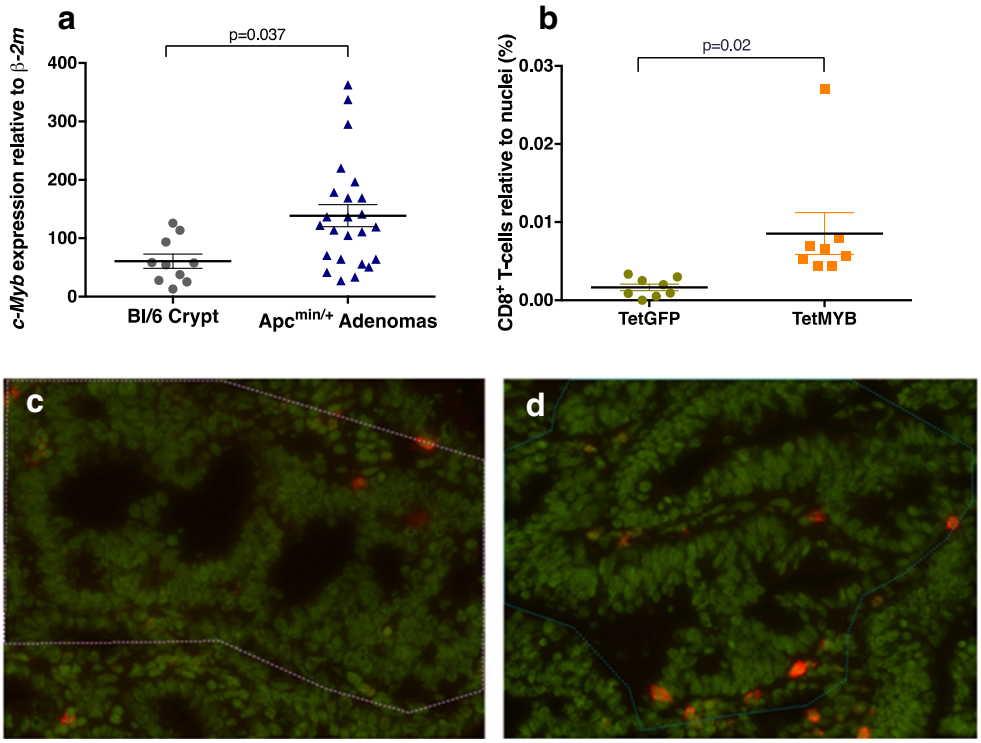

Fig. 3 c-Myb overexpression in $Apc^{min/+}$ colon adenomas was confirmed using qRT-PCR (**a**) (Student's *t* test). (**b**) TetMYB vaccination resulted in significantly more $CD8^+$ T cells infiltration at adenoma sites of $Apc^{min/+}$ mice than TetGFP on immunofluorescence (**c**) & (**d**) (Student's *t* test). $CD8^+$ T cells are depicted in red and aberrant crypts and adenomas in green

vaccinated group ($n = 8$; mean 334 ± 103 days SD) showed a survival benefit of 134 days compared to the unvaccinated group ($n = 7$; 200 ± 82 days SD) ($p = 0.01$) (Fig. 4). Interestingly, TetMYB vaccinated Apc580S mice did not only survive 50 days longer than the TetMYB $Apc^{min/+}$ mice, but also did not show signs of intestinal tumors as implied by as blood in their feces, anemia, weight loss, or a hunched stature. Similar to the $Apc^{min/+}$ mice study, adenomas of Apc580S mice, which reached the endpoint, were analyzed with qRT-PCR and immunohistochemistry staining for *c-Myb* expression and compared to normal C57BL/6 intestinal crypts. Our results confirm that mRNA as well as protein-levels of *c-Myb* are overexpressed in Apc580S adenomas when compared to normal C57BL/6 intestinal crypts ($p = 0.006$) (Fig. 5a, b). For verification of adenoma morphology, hematoxylin and eosin and β-catenin staining were performed on the Apc580S adenomas (Fig. 5c, d).

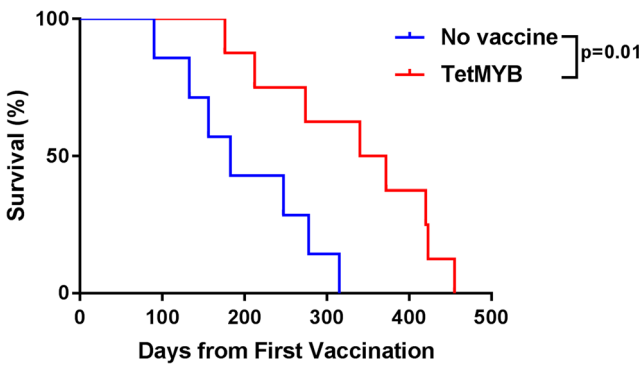

Fig. 4 Prophylactic TetMYB confers significant survival advantage over unvaccinated Apc580S mice (log-rank (Mantel-Cox) test)

Therapeutic TetMYB Vaccination Delayed Adenoma Progression

The prophylactic pre-clinical studies shown above forms the basis of using the TetMYB vaccine in high-risk patients before adenoma formation. Next, we explore the vaccine's efficacy in a therapeutic setting, i.e. against established adenomas. For this, we monitored adenoma growth by colonoscopic surveillance. Upon adenoma detection, Apc580S mice underwent 6 TetMYB vaccinations on a weekly basis. Anti-PD-1 antibody therapy was administrated simultaneously with the first 4 TetMYB vaccinations. We found that the intervention group ($n = 5$) had a statistically significant ($p < 0.0005$) reduction in adenoma growth rate compared to the untreated group ($n = 4$) (Fig. 6). These results demonstrate that the TetMYB vaccine in combination with anti-PD-1 antibody can potentially halt adenoma growth even therapeutically.

Effect of Prophylactic and Therapeutic TetMYB Vaccination on Adenoma Numbers

When Apc580S mice were culled at endpoint, we examined the number of adenomas in the colon. In the prophylactic experiment, we noted that the vaccinated group had numerically less adenomas than the untreated group however this was not statistically significant (Fig. 7a). This demonstrates that prophylactic intervention can *potentially* reduce adenoma burden.

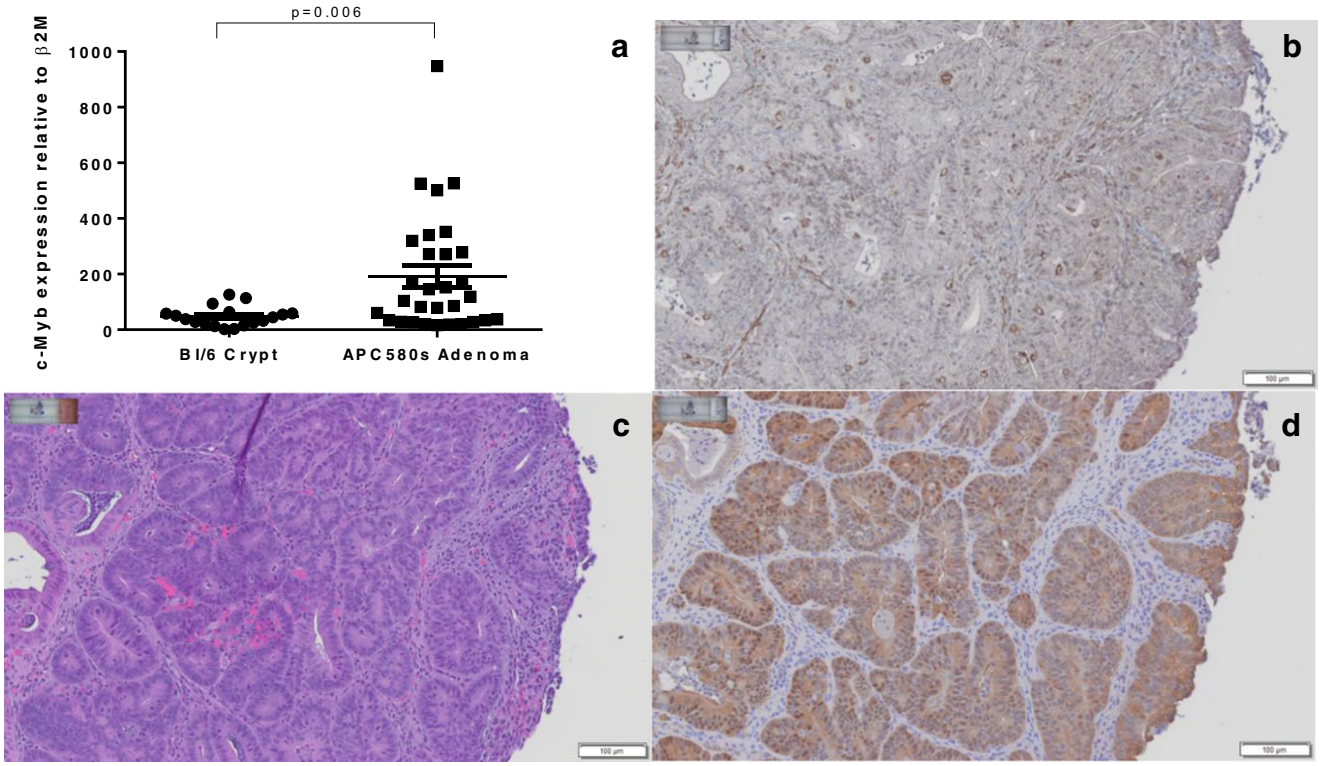

Fig. 5 Confirmation of *c-Myb* mRNA overexpression in Apc580S adenomas using qRT-PCR (**a**) (Student's *t* test). c-Myb (**b**), hematoxylin and eosin (**c**), and nuclear β-catenin (**d**) immunohistochemistry staining was used to verify adenoma status

The overall adenoma burden in all treatment groups of the therapeutic experiment was greater when compared to the prophylactic experiment because treatment was commenced after establishment of adenoma. Although there was no difference in the adenoma burden between the untreated and treated groups of therapeutic experiment, there was a reduction in adenoma growth rate in the treated group as demonstrated by the delayed progression to luminal obstruction (Fig. 6).

Discussion

Cancer vaccines represent an approach for the generation of tumor specific T cells in poorly immunogenic tumors, either against neo-antigens or differentially overexpressed self-antigens; such as MYB in this study. Most cancer vaccines have minimal adverse effects; however, to date, they have also demonstrated limited clinical benefit[23] due to immune

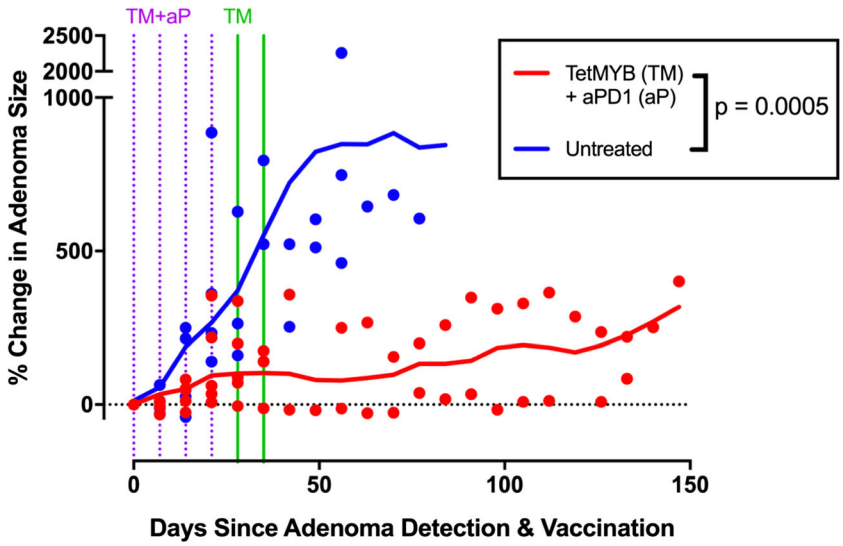

Fig. 6 Adenoma growth vs elapsed time since first detection. Immunotherapy interventions, TetMYB with anti-PD-1 antibody (purple) and TetMYB alone (green), results in significant reduction in adenoma growth rate (Student's *t* test)

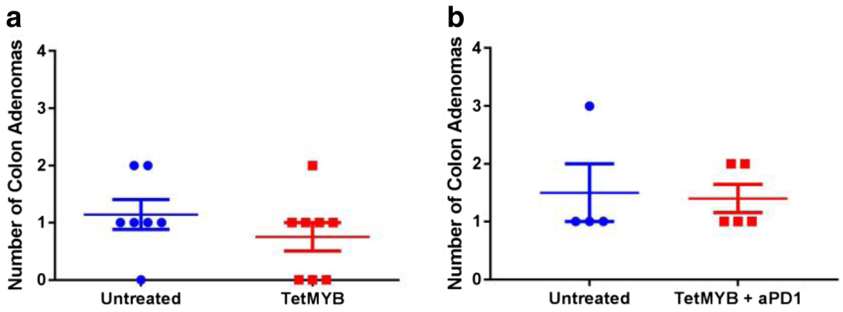

Fig. 7 Adenoma burden in colon from culled animals shows no significant difference between the two groups in both prophylactic (**a**) and therapeutic (**b**) experiments

suppressive mechanisms, including immune checkpoint expression, within the tumor microenvironment. However, the combination of immune checkpoint inhibitors with cancer vaccines is now showing first promising results.[24,25]

The concept of cancer-prevention vaccine is not novel, with notable successes in hepatocellular and cervical cancers using hepatitis B virus and human papilloma virus vaccines, respectively.[26] Nor is vaccinating against adenoma, with one trial using anti-MUC1 peptide vaccine for this purpose.[12] However, the MUC1 vaccine produced an antibody-mediated immune response, which is not ideal against colorectal cancer with recent evidence suggesting that a cytotoxic cellular response is associated with superior outcomes.[10]

MYB's connection with cancer has been established over previous decades and has led to the classification of MYB as a bona fide oncoprotein by its upstream and downstream interaction with hallmark cellular pathways that have been implicated in carcinogenesis (Fig. 8).[15,27–29] Moreover, elevated MYB expression in CRC has been correlated to poor cytotoxic T cell infiltration.[30]

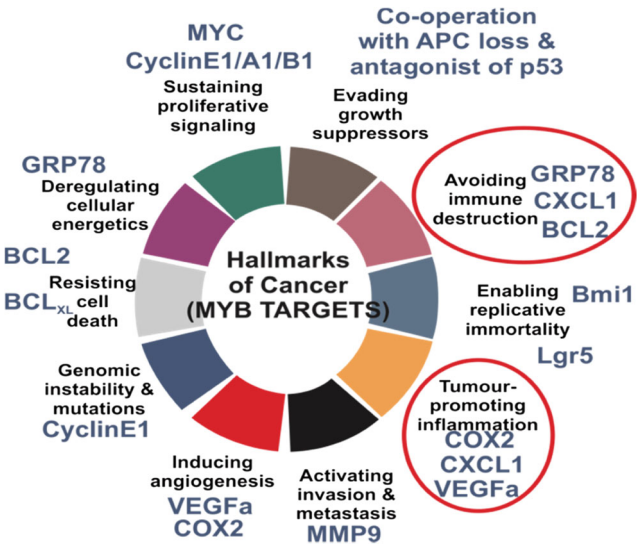

Fig. 8 MYB is a transcription factor that interacts with a range of carcinogenic genes (circled in red) that are encapsulated in the hallmarks of cancer (adapted from Hanahan and Weinberg, 2011)

Our study demonstrates that TetMYB vaccine is able to elicit a cytotoxic ($CD8^+$) T cell response, which is the hallmark of improved outcomes in CRC studies[31] and the basis of the recently validated Immunoscore.[10,32]

Furthermore, by eliciting cytotoxic immune responses against Myb in mouse models of sporadic colorectal adenoma (Apc580S) and FAP ($Apc^{min/+}$), we have demonstrated that our TetMYB vaccine conferred survival benefit prophylactically and therapeutically. The translational implication of these results is significant.

For patients with a personal history of adenomatous polyps, TetMYB vaccine has the potential to reduce the incidence of CRC by targeting interval or missed adenomas[33,34] and potentially lengthening colonoscopy interval thus reducing associated procedural risks as well as the burden on the health system. When given following curative CRC treatment, it may reduce the incidence of recurrence as well as fostering a less aggressive surveillance program with benefits to both patients and health system as stated prior. In the FAP population, TetMYB has the potential to delay the age of definitive proctocolectomy, or allow for rectal sparing surgery in cases with rectal adenomas, with both benefiting sexual function and family planning.[5]

We note here that progression into clinical trial will be the next proposed phase in the development of our TetMYB vaccine. It has been shown to be efficacious in pre-clinical models of CRC[14,19] which has led to a current first-in-human phase I trial: the MYPHISMO trial.[35] Should TetMYB prove to be safe and effective in the cancer therapy space, there will be an opportunity to conduct further prophylactic as well as therapeutic trials targeting high-risk groups as discussed above.

Limitations in our current study that will be addressed in future experiments include (i) utilizing colonoscopy to objectively assess adenoma formation in the prophylactic group to mitigate subjective bias when evaluating adenoma load using clinical signs, (ii) absence of TetGFP controls in the Apc580S therapeutic experiment, and (iii) evaluating the effect of anti-PD-1 antibody alone on adenomas. However, in the context of colorectal cancer, anti-PD-1 therapy alone is only effective in microsatellite unstable colorectal cancer,[8] which these mouse models do not emulate. We have also previously shown that anti-PD-1 monotherapy has little or no survival benefit in the MC38 and CT26 CRC mouse models.[14]

Conclusion

TetMYB has shown promising anti-adenoma activity in two mouse models of de novo colonic adenoma, which are mimicking the "classical" chromosomal instability pathway that represents >80% of CRC. Therefore, testing the TetMYB vaccine in a CRC prevention clinical trial targeting adenoma appears very promising and is currently being considered.

Author Contribution TP, SC, SS, JD, AGH, and RGR produced the study concept and design. TP, SC, and SS performed the scientific literature search and summarized all relevant studies. LP, SC, and TP manufactured the vaccines. TP, SC, and SS were involved in the data collection and analysis. PD provided the anti-PD-1 antibody. All authors were involved in refining the study design and data interpretation. TP, SC, SS, JD, PD, AGH, and RGR wrote the initial draft, with *all authors* involved in the editing process and final version of the manuscript. TP, SC, SS, and RGR compiled the tables, figures, and supplementary protocols and data.

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