Human perforin mutations and susceptibility to multiple primary cancers

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Loss-of-function mutations in the gene coding for perforin (*PRF1*) markedly reduce the ability of cytotoxic T lymphocytes and natural killer cells to kill target cells, causing immunosuppression and impairing immune regulation. In humans, nearly half of the cases of Type 2 familial hemophagocytic lymphohistiocytosis are due to bi-allelic *PRF1* mutations. The partial inactivation of PRF1 due to mutations that promote protein misfolding or the common hypomorphic allele coding for the A91V substitution have been associated with lymphoid malignancies in childhood and adolescence. To investigate whether *PRF1* mutations also predispose adults to cancer, we genotyped 566 individuals diagnosed with melanoma (101), lymphoma (65), colorectal carcinoma (30) or ovarian cancer (370). The frequency of *PRF1* genotypes was similar in all disease groups and 424 matched controls, indicating that the *PRF1* status is not associated with an increased susceptibility to these malignancies. However, 4 out of 15 additional individuals diagnosed with melanoma and B-cell lymphoma during their lifetime expressed either PRF1^{A91V} or the rare pathogenic PRF1^{R28C} variant (p = 0.04), and developed melanoma relatively early in life. Both PRF1^{A91V}- and PRF1^{R28C}-expressing lymphocytes exhibited severely impaired but measurable cytotoxic function. Our results suggest that defects in human PRF1 predispose individuals to develop both melanoma and lymphoma. However, these findings require validation in larger patient cohorts.

Introduction

Perforin (PRF1) is a pore-forming protein that is critical for the function of cytotoxic lymphocytes (CLs), which kill not only transformed cells but also cells harbouring intracellular pathogens.[1] CLs span both the innate and adaptive immune compartments, and comprise cytotoxic T lymphocytes (CTLs), natural killer (NK) cells, NKT cells and γδ T cells. These cells can secrete PRF1constitutively or in response to a pathogenic ("danger") signal, and most often do so together with serine proteases (granzymes), which synergize with PRF1 to mediate the death of target cells. PRF1 and granzymes are co-stored in acidic secretory vesicles and—upon the exocytosis of these granules—diffuse across the immune synapse to reach target cells.[2]

The fact that PRF1 is critical in both humans and mice can be gauged from the spectrum of pathologies associated with its deficiency.[2] Targeted *Prf1*-disruption in mice results in severe immunosuppression, which manifests primarily as an increased susceptibility to multiple viruses and (mostly intracellular) bacteria.[3] In addition, *Prf1*$^{-/-}$ mice succumb more readily to transplanted,[4] virus-induced[5] and spontaneous malignancies[6] than their wild-type (WT) counterparts. Specifically, more than 60% of Prf1-deficient mice develop aggressive B-cell lymphomas

beyond the age of 12 mo[6] and are more susceptible to sarcomas induced by the chemical carcinogen methylcholanthrene.[4] Congenital PRF1 deficiency in humans has been described for the first time relatively recently. Sporadic inactivating mutations of *PRF1* are infrequent, with a rate of heterozygosity of 1 in every 150 individuals in outbred populations.[7] Accordingly, bi-allelic mutations are rare but still account for around 50% of cases of the autosomal recessive disorder known as Type 2 familial hemophagocytic lymphohistiocytosis (FHL), which affects 1 in 90,000 live births.[8]

Children affected by Type 2 FHL and null for PRF1 activity present in early infancy (< 6 mo of age) with a profound immunoregulatory disorder, the hallmarks of which are pancytopenia, hemophagocytosis in the spleen and bone marrow, intractable fevers, neurological and renal dysfunction and markedly raised levels of circulating cytokines.[9] This condition is generally fatal unless the immune system is reconstituted through allogenic stem cell transplantation.[10,11] Conversely, in cases in which missense *PRF1* mutations allow for (some) residual PRF1 activity, the clinical presentation can be delayed to adolescence or even adulthood, and the manifestations may be atypical.[2] Many of these individuals present indeed with hematological cancers, intractable viral infections (in particular by the Epstein-Barr

Table 1. Frequency of perforin mutations in melanoma patients

Tumor group	Samples, n	A91V, n	Other variants, n	A91V variant, %	Variant (total), %	Fisher's test
Control	118	9	0	7.6	7.6	N/A
Melanoma	143	14	2*	9.8	11.2	p = 0.40
Melanoma + lymphoma	15	2	2	13.3	26.7	p = 0.04
Melanoma + other	28	3	0*	10.7	10.7	p = 0.71

*The N252S polymorphism, which has no apparent effect on perforin function in vitro, was found in a third patient but s/he was not included in the statistical calculations.

virus) or post-viral syndromes such as the polyneuropathy known as Guillain-Barre syndrome.[12]

A number of studies have previously examined the genotype of *PRF1* in cohorts of patients affected by hematological malignancies to test whether an association would exist between the relatively common allele encoding PRF1[A91V] (in which the alanine residue at position 91 is substituted by valine) and cancer susceptibility. *PRF1[A91V]* heterozygotes comprise 8–9% of healthy Caucasian populations, although far fewer African Americans and Japanese carry this allele.[13,14] Surprisingly, it has recently been shown that the A91V substitution, which was previously assumed to be biochemically "conservative" and therefore of little functional importance, causes a severe reduction in PRF1 activity.[15] These findings have added some substance to the observation that A91V is "overrepresented" in the few cases of late onset Type 2 FHL identified to date, particularly in a homozygous state. A study conducted on a small patient cohort has identified a tentative relationship between A91V and childhood leukemia/lymphoma.[16] This association was not confirmed by a subsequent study involving a larger patient cohort, yet A91V turned out to be more common in a subgroup of 24 patients bearing BCR-ABL[+] acute lymphoblastic leukemia.[13] Appropriately, the authors recommended caution in assigning biological significance to this observation, due to the small sample size.

Here, we performed a retrospective analysis of the incidence of the A91V-coding allele and other PRF1 mutations in adults diagnosed with a variety of hematological or epithelial malignancies and appropriately matched healthy subjects. Although we found no increased prevalence of *PRF1[A91V]*, the frequency of *PRF1* mutations was elevated in melanoma patients who had also experienced a second malignancy in their lifetime, especially a B-cell lymphoma. Four unrelated patients out of 15 found in this subset inherited either *PRF1[A91V]* or a rare *PRF1* allele (*PRF1[R28C]*), which we demonstrate here to code for a PRF1 variant that has minimal lytic activity, and hence to represent a second but genuinely defective *PRF1* allele.

Results

In order to investigate a possible association between the severely hypomorphic allele *PRF1[A91V]* and adult cancer, we genotyped 566 individuals who had been diagnosed with melanoma, lymphoma, colorectal or ovarian cancer. We have previously shown that the A91V substitution, which has a frequency of 8–10% in various Caucasian populations, results in severe PRF1 dysfunction.[19] We found that the frequency of *PRF1[A91V]* heterozygosity in an independent, healthy, gender-matched control population was 7.6% (9/118), which was not statistically different from the *PRF1[A91V]* frequency observed among 30 colorectal cancer (2/30, 6.7%) or ovarian cancer (21/370, 5.7%) patients (**Table 1**; **Table S1**). There was no over-representation of *PRF1[A91V]* in either of the major histological subtypes of ovarian cancer, mucinous or serous (data not shown). The incidence of *PRF1[A91V]* in a further group of women diagnosed with various benign ovarian pathologies was slightly elevated (25/241, 10.4%) in comparison to that of ovarian cancer patients, but this did not reach statistical significance (p < 0.07).

We tested 143 patients that had been diagnosed with malignant skin melanoma, an immunogenic cancer that has never been analyzed with respect to *PRF1* mutations. Fourteen patients (9.8%) were positive for the A91V substitution, not significantly more than the control healthy population (7.6%) (**Table 1**). We were surprised to find 2 patients bearing a heterozygous mutation encoding PRF1[R28C], which has recently been described for the first time in a patient affected by juvenile rheumatoid arthritis and the macrophage activation syndrome.[20] As the original report had not characterized PRF1[R28C] functionally, we studied this PRF1 variant in a number of in vitro cytotoxicity assays. Using standard 4 h ^{51}Cr release assays to quantify target cell death, we found that primary cytotoxic T lymphocytes from *Prf1^{-/-}* mice expressing PRF1[R28C] exhibit a dramatic functional impairment (> 80%) as compared with the same cells expressing WT PRF1 (**Fig. 1**).

Including *PRF1[R28C]* in the analysis brought the overall incidence of *PRF1* variants in the melanoma patient cohort to 11.2% (16/143, **Table 1**). When we checked the annotated clinical histories of all these patients, we noted that both *PRF1[R28C]*-bearing individuals as well as 2 *PRF1[A28V]*-bearing subjects had also experienced B-cell lymphoma. Our melanoma patient cohort included a total of 15 individuals who had experienced both melanoma and B-cell lymphoma, and 4/15 (26.7%) of these subjects were heterozygous for a pathological PRF1 mutation, be it A91V or R28C (p = 0.04). We identified additional 28 patients who had melanoma and a second primary malignancy other than B-cell lymphoma, 3 of whom were positive for A91V while 1 carried PRF1[N252S], which has previously been reported to have normal activity.[19] Globally, *PRF1* mutations were hence identified in 8 out of 43 subjects who had experienced melanoma and another malignancy, corresponding to 18.6% of this patient subgroup (p = 0.08).

Among patients diagnosed with 2 cancers including a melanoma, the time interval between the 2 diagnoses widely varied
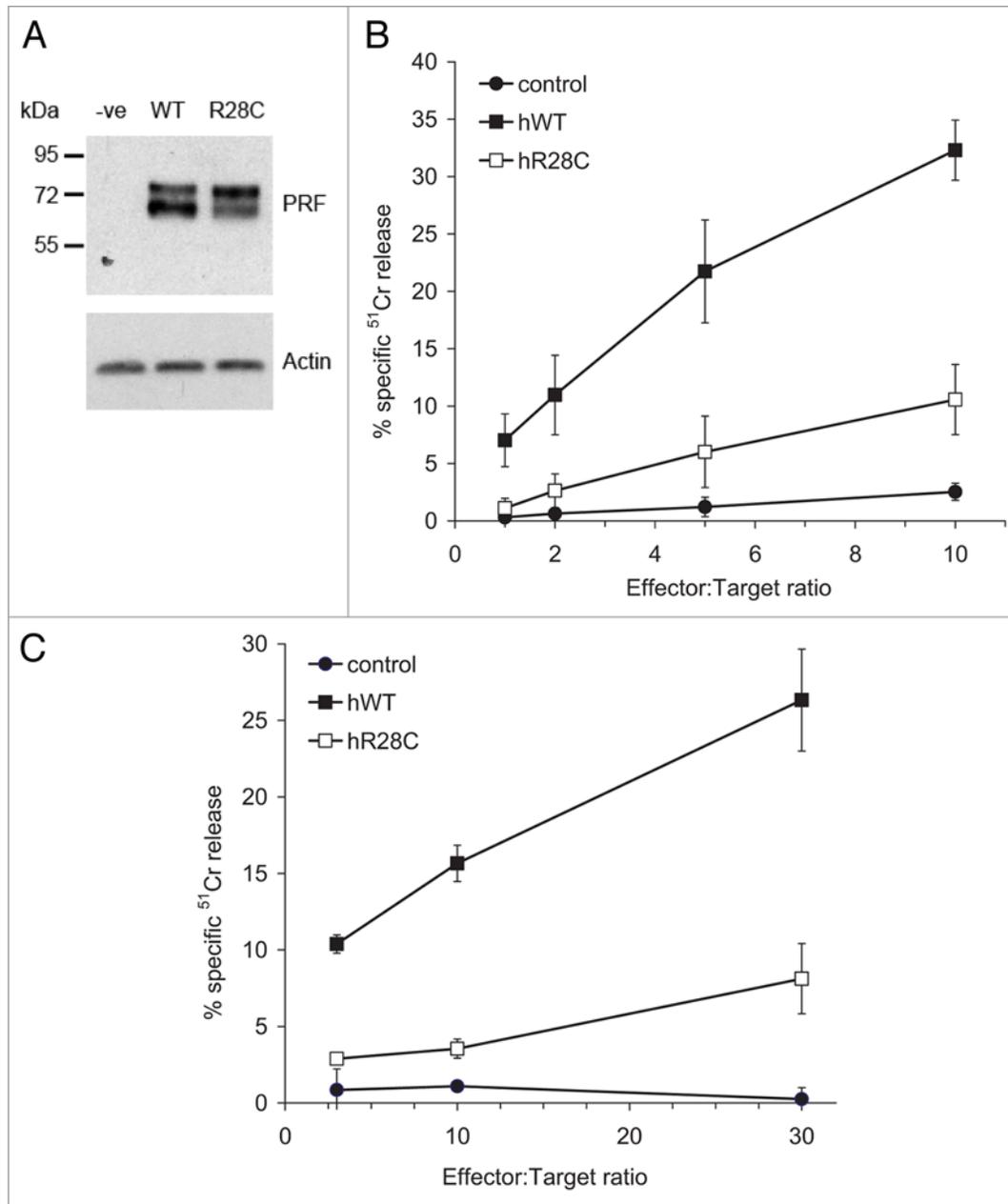
Figure 1. Effect of R28C mutation on perforin function. The gel panel shows a western blot for perforin levels in transiently-transfected RBL cells (**A**). Results of representative cytotoxicity assays are shown for a RBL cytotoxicity assay (**B**), and an assay using CD8^{+} T cells from *Prf1*^{−/−}.*OT-1* mice (**C**). Data points represent the mean values from 3 experiments ± standard error of the mean. In all of these experiments, effector cells were electroporated with human wild-type or R28C mutant *PRF1* cloned into pIRES2-eGFP expression vector. Transfected cells were isolated on the basis of identical eGFP fluorescence and their cytotoxic activity against 51Cr labeled and trinitrobenzosulfonic acid-labeled Jurkat cells (in the case of RBL effectors) or SIINFEKL (ova) peptide pulsed EL-4 target cells (in the case of CD8^{+} Tcells), were tested as described in *Materials and Methods*.

between a synchronous diagnosis and 25 y, and a similar proportion of patients developed melanoma as the first or second malignancy (48.5% or 51.5%, respectively). This was also the case for individuals diagnosed with both melanoma and B-cell lymphoma during their lifetime. However, it was interesting to note that melanoma was diagnosed relatively early (mean = 44.8 y, n = 4) in patients bearing a *PRF1* mutation as compared with individuals carrying WT *PRF1* in homozygosity (mean = 63.3 y, n = 11). By contrast, hematological malignancies developed at a similar age irrespective of whether the patients carried a *PRF1* mutation (mean = 56.8 y) or not (mean = 59.6 y).

Discussion

The specific and pronounced susceptibility of *Prf1*^{−/−} mice to develop spontaneous B-cell lymphomas indicates that, at least in mice, the immune system in general and CLs in particular play an important role in eliminating transformed cells before tumors

become clinically evident. The rarity of FHL and the fact that PRF1-deficient children do not often survive infancy has made it very difficult to ascertain whether a similar mechanism also exists in humans, although previous studies have found evidence in support of this contention.[13,16] We have previously shown that PRF1 is likely to protect humans against hematological cancers, as of all non-consanguineous patients recorded in the literature bearing bi-allelic *PRF1* mutations including *PRF1^A91V^* (n = 26) but remaining disease-free to the age of > 10 y, 50% presented with a spectrum of hematological cancers.[12]

The current study aimed to examine the incidence of mono-allelic *PRF1^A91V^* and when possible, other *PRF1* mutations in populations of adult individuals affected by lymphoma or epithelial malignancies. We elected to study several hundred patients previously diagnosed with melanoma, lymphoma, ovarian cancer or colorectal cancer, diseases in which there is independent evidence in support of a role for CD8$^+$ T cells in the prevention of disease progression and/or metastasis. Melanoma has long been recognized as an "immunogenic" cancer, one of the few human malignancies in which circulating CTLs with overt antitumor activity ex vivo can be easily isolated.[21,22] In both ovarian and colorectal carcinoma, tumor infiltration by CD8$^+$ lymphocytes has been associated with a favorable patient prognosis.[23] This is particularly the case for colorectal cancer, in which the degree of lymphocytic infiltration has been claimed to be a more reliable predictor of overall survival than cancer stage at the time of diagnosis.[24] We found a marginal increase in the frequency of *PRF1* mutations among 143 melanoma patients (11.9%, comprising 14 *PRF1^A91V^*-positive individuals, 2 patients bearing the rare *PRF1^R28C^* allele and 1 subject carrying *PRF1^N252S^*), as compared with a healthy, gender-matched control population. Still, the *PRF1* mutation appeared to be particularly enriched in melanoma patients who had also been diagnosed with a second malignancy, mainly B-cell lymphoma. Although this was a small sub-group, melanoma occurred at a relatively early age among the carriers of mutant *PRF1*, supporting the notion that PRF1 mutations negatively influence immunosurveillance, hence accelerating the development of clinically manifest lesions.

In our opinion, it is unlikely that the treatment for melanoma predisposed patients to B-cell lymphoma or vice versa. Surgical excision, interferon treatment (rarely used) and radiotherapy are indeed unlikely to cause any of the second malignancies seen among melanoma patients, and the chemotherapy for advanced melanoma does not involve anthracyclines (which have previously been implicated in the development of secondary acute myeloid leukemia). Along similar lines, there is no therapeutic approach for hematological malignancies known to predispose to melanoma, exception made—perhaps—for therapy-related immunosuppression itself, which is transient in successfully treated individuals.

How then, in a mechanistic sense, could mono-allelic *PRF1* mutations adversely affect cancer immunosurveillance, given a co-dominant allelic expression? We have previously shown that PRF1^A91V^ can exert a "dominant negative" effect, that is, it can substantially reduce the activity of WT PRF1 with which it is

packaged in CL granules.[15] As granzymes are entirely dependent on PRF1 to effect apoptosis,[25] it is possible the presence of PRF1^A91V^ might substantially reduce the overall cytotoxic activity of CLs, even when WT PRF1 is co-expressed. While evidence in support of this hypothesis is still lacking, it is also possible that polymorphisms affecting the large and complicated *PRF1* promoter/enhancer might impact negatively on PRF1 as produced by the WT allele, so that PRF1^A91V^ or PRF1^R28C^ would be synthetized in relative excess. Further studies are needed to investigate this possibility.

This is the first report linking a partial loss of PRF1 activity to a non-hematological neoplasm, melanoma. Given the small number of patients bearing dual primary cancers included in our study, we look forward to other groups testing our conclusions in independent patient cohorts. As CLs play a critical role in killing virus-infected cells, we are also interested in determining whether PRF1 defects affect the incidence or clinical course of malignancies that have a viral etiology.

Materials and Methods

Patients and control subjects. Unless otherwise stated, all patients attended either the Peter MacCallum Cancer Centre (Peter Mac) or the Oncology Department of Austin and Repatriation Medical Centre, 2 large tertiary referral centers in Melbourne (Australia). DNA from healthy control subjects was sourced from the Peter Mac Biobank. Ovarian cancer cases and controls were residents of Southampton, UK between 1993 and 1998, as described previously,[17] and comprised representative numbers of serous, mucinous, endometrioid, clear cell and undifferentiated adenocarcinomas. All of the controls were white female volunteers or obstetrics outpatients. The control and cancer groups were drawn from the same geographical area and were predominantly Anglo-Saxon. Surgically excised colon carcinomas were collected at Western General Hospital, Melbourne, Australia, between 1993 and 1999. The collection of all bio-specimens and patient data was approved by the respective human ethics committees, and their use in the current study was approved by Peter Mac's Human Ethics Committee.

Preparation of genomic DNA. Clinical samples were received as whole blood, tumor tissue or (occasionally) pre-purified genomic DNA. For DNA purification, leukocytes or tissue were digested overnight at 55°C in extraction buffer (100 mM Tris pH 8.0, 5 mM EDTA, 0.5% sodium dodecyl sulfate, 200 mM NaCl) containing 10 mg/mL proteinase K. Residual debris were removed by centrifugation and genomic DNA precipitated by adding an equal volume of ice-cold isopropanol. The DNA pellet was washed with 70% ethanol, air-dried and redissolved in sterile water.

High resolution melt (HRM). HRM was performed in a 2-plex QIAGEN Rotor-Gene Q apparatus (QIAGEN Australia), to detect *PRF^A91V^*. The primers used were F653: 5'-GGC CCG CCA GTT GGT GAG-3' and R638: 5'-CAC CCT CTG TGA AAA TGC CCT ACA G-3', producing a product of 85 bp, which was analyzed using QIAGEN Rotor-Gene Q software version 2.0.3.

DNA sequencing. *PRF1* exons were amplified using the following primers: Exon 2 (1827: 5'-CCC CTG TCT CTG CAG

CTC-3', 1830: 5'-CCC TAG CCC CAG CTC TCA-3'); first portion of Exon 3 (3F: 5'-CCA GTC CTA GTT CTG CCC ACT TAC-3', 1836: 5'-CAT GCT TGG ATG AAG GTC AC-3'); second portion of Exon 3 (1835: 5'-CCT CCA TTA ACG ACC TGC TG-3', 3R: 5'-GAA CCC CTT CAG TCC AAG CAT AC-3'), and then gel purified. The same primers were used for sequencing, based on the ABI PRISM BigDye Terminator v3.1 Cycle Sequencing (Applied Biosystems).

PRF1 expression and function. WT and mutant PRF1[R28C] (generated by site-directed mutagenesis[18]) were expressed in rat basophilic leukemia (RBL) cells or in primary cytotoxic T lymphocytes of *Prf1*^{–/–} mice that were also transgenic for an ovalbumin-specific T-cell receptor (*Prf1*^{–/–}*.OT-1* mice) and cytotoxicity was assessed as described previously.[15]

Statistical analyses. The frequency of *PRF1* mutations in melanoma patients was compared with healthy control samples using two-tailed Fisher's exact test. In the case of ovarian cancer, the equivalent comparison was made with a control population with benign ovarian conditions collected in the same geographic area (Southampton, UK).

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Supplemental Materials

Supplemental material may be found here: http://www.landesbioscience.com/journals/oncoimmunology/article/24185/

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