Human perforin mutations and susceptibility to multiple primary cancers

Joseph A. Trapani,1,2,* Kevin Y.T. Thia,1 Miles Andrews,3 Ian D. Davis,3 Craig Gedye,3 Philip Parente,4 Suzanne Svobodova,3 Jenny Chia,1 Kylie Browne,1 Ian G. Campbell,1 Wayne A. Phillips,3 Illia Voskoboinik1 and Jonathan S. Cebon2

1Cancer Immunology Program; Peter MacCallum Cancer Institute; East Melbourne, VIC Australia; and Sir Peter MacCallum Department of Oncology; The University of Melbourne; Melbourne, VIC Australia; 2Research Division; Peter MacCallum Cancer Centre; East Melbourne, VIC Australia; 3Ludwig Institute for Cancer Research; Austin Hospital; Heidelberg, VIC Australia; 4Department of Medical Oncology; Box Hill Hospital; VIC Australia

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 hypomorphitic 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 PRF1A91V or the rare pathogenic PRF1R28C variant (p = 0.04), and developed melanoma relatively early in life. Both PRF1A91V- and PRF1R28C-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.

Keywords: perforin, melanoma, lymphoma, dual tumors, A91V, FHL

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 PRF1 constitutively 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-induced5 and spontaneous malignancies6 than their wild-type (WT) counterparts. Specifically, more than 60% of Prf1-deficient mice develop aggressive B-cell lymphomas beyond the age of 12 mo6 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
susceptibility.
ine residue at position 91 is substituted by valine) and cancer
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PRF1A91V
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B-cell lymphoma. Four unrelated patients out of 15 found in this
experienced a second malignancy in their lifetime, especially a
PRF1
malignancies to test whether an association would exist between the
the A91V substitution, which has a frequency of 8–10% in
Caucasian populations, although far fewer African Americans
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 homo-
zygous 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 bear-
ing BCR-ABL+ acute lymphoblastic leukemia.17 Appropriately,
the authors recommended caution in assigning biological signifi-
cance 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 malign-
ancies and appropriately matched healthy subjects. Although
we found no increased prevalence of PRF1A91V, 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 PRF1A91V or a rare PRF1 allele (PRF1R28C),
which we demonstrate here to code for a PRF1 variant that has
minimal lytic activity, and hence to represent a second but genu-
inely defective PRF1 allele.

Results

In order to investigate a possible association between the severely
hypomorphic allele PRF1A91V and adult cancer, we genotyped
566 individuals who had been diagnosed with melanoma, lymph-
oma, 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 dysfunc-
tion.19 We found that the frequency of PRF1A91V heterozygosity in
an independent, healthy, gender-matched control population
was 7.6% (9/118), which was not statistically different from the
PRF1A91V 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
PRF1A91V in either of the major histological subtypes of ovarian
cancer, mucinous or serous (data not shown). The incidence of
PRF1A91V 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 muta-
tion encoding PRF1R28C, which has recently been described for
the first time in a patient affected by juvenile rheumatoid arthri-
tis and the macrophage activation syndrome.20 As the original
report had not characterized PRF1R28C functionally, we studied
this PRF1 variant in a number of in vitro cytotoxicity assays. Using
standard 4 h ⁵¹Cr release assays to quantify target cell
death, we found that primary cytotoxic T lymphocytes from
Prf1−/− mice expressing PRF1R28C exhibit a dramatic functional
impairment (> 80%) as compared with the same cells expressing
WT PRF1 (Fig. 1).

Including PRF1R28C in the analysis brought the overall inci-
dence of PRF1 variants in the melanoma patient cohort to 11.2%
(16/143, Table 1). When we checked the annotated clinical his-
tories of all these patients, we noted that both PRF1R28C-bearing
individuals as well as 2 PRF1A91V-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
PRF1N252S, 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 mela-
noma, the time interval between the 2 diagnoses widely varied

| 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.

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 |
|-------------|------------|---------|------------------|----------------|-------------------|--------------|
| Melanoma    | 143        | 10      | 1*               | 7.0            | 7.0               | N/A          |
| Melanoma + lymphoma | 15    | 2       | 2                | 13.3           | 26.7              | p = 0.04     |
| Melanoma + other | 28    | 3       | 0*               | 10.7           | 10.7              | p = 0.71     |
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.\textsuperscript{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\textsuperscript{A91V} (n = 26) but remaining disease-free to the age of > 10 y, 50% presented with a spectrum of hematological cancers.\textsuperscript{12}

The current study aimed to examine the incidence of mono-allelic PRF1\textsuperscript{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\textsuperscript{+} 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.\textsuperscript{21,22} In both ovarian and colorectal carcinoma, tumor infiltration by CD8\textsuperscript{+} lymphocytes has been associated with a favorable patient prognosis.\textsuperscript{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.\textsuperscript{24} We found a marginal increase in the frequency of PRF1 mutations among 143 melanoma patients (11.9\%), comprising 14 PRF1\textsuperscript{A91V}-positive individuals, 2 patients bearing the rare PRF1\textsuperscript{R28C} allele and 1 subject carrying PRF1\textsuperscript{P252S}, 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\textsuperscript{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.\textsuperscript{15} As granzymes are entirely dependent on PRF1 to effect apoptosis,\textsuperscript{25} it is possible the presence of PRF1\textsuperscript{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\textsuperscript{A91V} or PRF1\textsuperscript{R28C} would be synthesized 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,\textsuperscript{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 PRF1\textsuperscript{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 GTT GGT GAG-3′ and R638: 5′-CAC CCT CTG TGA AAA TGC CCT ACA G-3′) using QIAGEN Rotor-Gene Q software version 2.0.3.
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.

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

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