**Human Cancer Biology**

**BRAF/NRAS Wild-Type Melanomas Have a High Mutation Load Correlating with Histologic and Molecular Signatures of UV Damage**

Victoria J. Mar1,10,13, Stephen Q. Wong2, Jason Li4, Richard A. Scolyer14,15,16, Catriona McLean10,11, Anthony T. Papenfuss7,8, Richard W. Tothill4, Hojabr Kakavand14,15,16, Graham J. Mann15, John F. Thompson14,15,16, Andreas Behren12, Jonathan S. Cebon12, Rory Wolfe13, John W. Kelly10, Alexander Dobrovic2,6, and Grant A. McArthur1,3,5,6,9

**Abstract**

**Purpose:** The mutation load in melanoma is generally high compared with other tumor types due to extensive UV damage. Translation of exome sequencing data into clinically relevant information is therefore challenging. This study sought to characterize mutations identified in primary cutaneous melanomas and correlate these with clinicopathologic features.

**Experimental Design:** DNA was extracted from 34 fresh-frozen primary cutaneous melanomas and matched peripheral blood. Tumor histopathology was reviewed by two dermatopathologists. Exome sequencing was conducted and mutation rates were correlated with age, sex, tumor site, and histopathologic variables. Differences in mutations between categories of solar elastosis, pigmentation, and BRAF/NRAS mutational status were investigated.

**Results:** The average mutation rate was 12 per megabase, similar to published results in metastases. The average mutation rate in severely sun damaged (SSD) skin was 21 per Mb compared with 3.8 per Mb in non-SSD skin ($P = 0.001$). BRAF/NRAS wild-type (WT) tumors had a higher average mutation rate compared with BRAF/NRAS–mutant tumors (27 vs. 5.6 mutations per Mb; $P = 0.0001$). Tandem CC>TG/GC>AA mutations comprised 70% of all dinucleotide substitutions and were more common in tumors arising in SSD skin ($P = 0.0008$) and in BRAF/NRAS WT tumors ($P = 0.0007$). Targetable and potentially targetable mutations in WT tumors, including NF1, KIT, and NOTCH1, were spread over various signaling pathways.

**Conclusion:** Melanomas arising in SSD skin have higher mutation loads and contain a spectrum of molecular subtypes compared with BRAF- and NRAS-mutant tumors indicating multigene screening approaches and combination therapies may be required for management of these patients. *Clin Cancer Res;* 19(17); 4589–98. ©2013 AACR.

**Introduction**

The development of BRAF inhibitors, vemurafenib and dabrafenib, marked a turning point in the treatment and prognosis of patients with advanced stage metastatic melanoma. The BRAF oncogene is mutated in up to 50% of cutaneous melanomas and its clinical and histologic associations have been well characterized (1, 2). BRAF V600E mutations occur more commonly in patients under the age of 50 years with higher nevus counts, and are more common in melanomas arising on the trunk in intermittently sun-exposed skin (1, 3). They are also more prevalent in superficial spreading melanomas (SSM) as compared with other melanoma subtypes and have characteristic histopathologic features (1, 4, 5). In contrast, BRAF V600K mutations are...
Translational Relevance

Targeted therapy directed at the oncogene, BRAF, has improved melanoma patient outcome in recent years. Targeted strategies for NRAS-mutant melanoma are currently under investigation. Here, we show that BRAF/NRAS wild-type (WT) tumors are a complex group, more commonly arising in sun-exposed sites associated with severe solar elastosis. Consistent with this, they have a high mutation load with a large proportion of C>T transitions as well as dinucleotide CC>TT transitions specific for UV-induced damage. It is likely that a number of genomic insults are required cumulatively for melanoma progression in this group. Potentially targetable mutations in BRAF/NRAS WT melanomas are spread over several different signaling pathways and this may in the future have implications for therapeutic approaches to patients harboring such tumors. Classification of melanoma into BRAF-mutant, NRAS-mutant, and high-mutation load groups may assist in identification of patients more likely to respond to particular combined drug therapies.

Materials and Methods

Selection of samples for discovery phase

Fresh-frozen tissue from primary cutaneous melanomas was collected prospectively from melanoma clinics at the Victorian Melanoma Service, Alfred Hospital (Prahran, VIC, Australia) and Peter MacCallum Cancer Centre (East Melbourne, VIC, Australia; n = 5) as well as retrospectively from the Victorian Cancer Biobank (n = 8) and Melanoma Institute Australia Biospecimen Bank (n = 21). Matched patient blood was also collected to distinguish somatic mutations from germline mutations. In one case, matched blood was not available, therefore normal DNA was extracted from dissected adjacent normal tissue (case NM002). All patients gave informed consent and ethics approval was obtained from the Peter MacCallum Cancer Centre Ethics Committee (11 of 25) for all human tissues and clinicopathologic data used in this study. The histopathology of all cases was reviewed by two dermatopathologists (C. McLean and R.A. Scolyer) and various histopathologic features were scored (detailed later). Thirty-two tumors were verified on hematoxylin and eosin stain as containing more than 80% of tumor. The remaining two samples (NM002 and NM019) contained 50% to 60% tumor respectively and were included as the sensitivity of mutation detection by next-generation sequencing remains high even for impure samples (18). Tumor and matched normal DNA was extracted using the QIagen Gentra Puregene Kit (Qiagen) according to the manufacturer’s protocol. Extracted DNA was quantified using a Qubit fluorometer (Invitrogen) and a Quant-it dsDNA HS kit (Invitrogen).

Assessment of clinical and histologic variables

Patient information including date of birth, date of surgery, gender, and site of tumor was collected. Histologic variables such as Breslow thickness (mm), ulceration, mitotic rate (n/mm²), melanoma subtype, presence of regression, lymphovascular invasion, neutropoikemia, and microsatellite lesions were assessed. Melanoma subtype was classified according to the World Health Organization (WHO) criteria into nodular melanoma (NM), SS, and lentigo maligna melanoma (LMM; ref. 19). Solar elastosis was graded (0, none; 1, mild; 2, moderate; 3, severe) according to the amount of elastotic fibers in normal skin adjacent to the melanoma in the excision specimen (3). Histologic evidence of solar damage was also dichotomized; tumors arising on non–severely sun damaged (non-SSD; solar elastosis scores of 0 or 1) and tumors arising on SSD skin (solar elastosis scores of 2 or 3). Pigmentation was graded according to the amount of pigment within constituent melanocytes (3). Tumor lymphocytic infiltration was graded 0–3 according to the density and distribution of the lymphocytic infiltrate within the dermal component of the tumor (20). Features of nesting, scatter, epidermal contour, and circumscription were also assessed (3).

Exome sequencing

A schematic summary of how samples were sequenced and analyzed is shown in Supplementary Fig. S1. One
A total of 34 primary cutaneous melanomas were sequenced from 34 patients (39% female) with a median age at diagnosis of 67 years. Melanomas were from the lower limb (47%), head and neck (26%), trunk (18%), and upper limb (9%). Fifty-three percent of cases arose in non-SSD skin. For each tumor, the number of mutations in eight pathways, well described in melanoma (30), was assessed. Gene(s) listed in each pathway are described in Table S3.

Validation of mutations

The BRAF and NRAS mutation status of each tumor was determined using high-resolution melting analysis (HRM) and Sanger sequencing as previously described (31).

Actionable mutations shown in Supplementary Table S4 were validated using Sanger sequencing. PCR amplification was conducted on a Rotor-Gene Q (Qiagen) or a LightCycler 480 (Roche Diagnostics). Primer sequences and specific PCR reaction conditions are available upon request. Following PCR amplification, a 1:10 dilution PCR product was sequenced using the BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems). The sequencing products were purified with Agencourt CleanSEQ beads (Beckman Coulter), followed by capillary electrophoresis on an ABI 3730 DNA Sequencing instrument (Applied Biosystems). Data analysis was conducted with Sequencher software, version 4.6 (Gene Codes).

Validation of mutation load in BRAF/NRAS WT tumors

To validate our concept of a high-mutation load group of melanomas, we retrospectively examined exome data from Hodis and colleagues (14) and Krauthammer and colleagues (15), which sequenced independent cohorts with a total of 198 cutaneous melanomas and described the mutation rate together with BRAF and NRAS status. Acral, mucosal, and uveal melanomas were excluded.

Statistical analysis

Nonparametric Spearman correlation was used to investigate associations between mutation rate and other continuous variables (thickness and mitotic rate) to avoid any undue influence of outliers. The Mann–Whitney U and Kruskal–Wallis tests were used to assess associations between mutation rate and respectively dichotomous variables and variables with three or more categories. Ordered logistic regression was used to assess associations between solar elastosis scores and continuous variables. Multinomial logistic regression was used to assess for associations between BRAF V600E, BRAF V600K, NRAS, and WT tumors. All analyses were conducted using Stata12 statistical software (Stata Corporation).

Results

A total of 139,962 somatic mutations located in canonical transcripts [the most prevalent transcript as detailed by the UniProt Knowledgebase (27)], with bidirectional read support, and mutations predicted to be potentially deleterious (mutations which potentially change the coding of a protein, i.e., nonsynonymous, splice site, indels, stop codon lost, and stop codon gain mutations). A list of potentially “actionable” mutations, predicted to have diagnostic, prognostic, or therapeutic implications, was generated by comparing our list of filtered mutations to potential targets listed by Catalogue of Somatic Mutations in Cancer (COSMIC; ref. 28) and the Genomics of Drug Sensitivity in Cancer Project (GDSC), Sanger Institute (Cambridgeshire, United Kingdom; ref. 29).

To assess the frequency of commonly mutated genes in this cohort, a literature search was conducted to identify mutations validated as occurring in more than 10% of melanomas.

Pathway analysis

For each tumor, the number of mutations in eight pathways, well described in melanoma (30), was assessed. Gene(s) listed in each pathway are described in Supplementary Table S3.

Validation of mutations in BRAF/NRAS Wild-Type Melanomas
with an average of 4,117 (range, 105–28,507) mutations per tumor (or on average 33 mutations/Mb). Of these, 37,981 mutations (12 mutations/Mb) were predicted to be deleterious (Fig. 1), consistent with rates of nonsynonymous changes reported in other studies (14, 15).

**Signatures of UV-induced damage**

The ratio of nonsynonymous to synonymous changes was 1.7, consistent with other melanoma exome studies (11, 32). A breakdown of mutational consequences based on the deleterious filter is shown in Fig. 2A. Eighty-five percent of SNVs were composed of C>T transitions, similar to results published elsewhere (10, 15). The UV signature of DNA damage was also confirmed by investigating dinucleotide CC>TT changes, which made up 73% of all dinucleotide substitutions (Fig. 2C). Tumors arising in SSD skin had an average of 26 CC>TT transitions, compared with an average of four in tumors arising in non-SSD skin (P = 0.0008). The number of CC>TT transitions as a percentage of total dinucleotide transitions in tumors with no solar elastosis was 36% compared with 74% in tumors with severe solar elastosis (P = 0.04; Fig. 2C). BRAF/NRAS WT tumors had an average of 34 CC>TT transitions compared with an average of six in both NRAS- and BRAF-mutant tumors (P = 0.0007).

Tumors which showed no CC>TT/GG>AA changes (n = 6) were from relatively sun-protected sites (such as the lower limb and trunk) with low solar elastosis scores (Fig. 2C) and were either BRAF- or NRAS-mutant. Only one of these tumors arose in SSD skin (solar elastosis score of 2) and was BRAF V600K-mutant.

**Correlation of mutation rate with clinical and histopathologic variables**

Melanoma arising on sun-exposed anatomic sites (head, neck, and upper limb) had higher mutation rates compared with those arising on the lower limb and trunk as would be expected, although this difference did not reach statistical significance (P = 0.07). Mutation rates were more than 5-fold in tumors arising in skin with severe solar elastosis compared with tumors arising in skin with no solar elastosis (P = 0.001; Fig. 3).

Interestingly there was an inverse correlation between thickness and mutation rate (r = −0.4; P = 0.02; Fig. 4). There was no statistically significant association between mutation rate and other clinicopathologic features such as age, sex, mitotic rate, tumor subtype, pigmentation, features of regression, or tumor-infiltrating lymphocytes.

**Mutation rate in tumors with classical melanoma mutations**

Thirty-five percent of tumors in the discovery cohort had a canonical BRAF V600E or V600K mutation and 35% were NRAS-mutant in exon 3 (Fig. 1). Interestingly, the proportion of BRAF V600K (7 of 34) mutations was greater than BRAF V600E mutations (5/34). All BRAF and NRAS mutations were independently confirmed by Sanger sequencing. Consistent with previous reports (6), most BRAF V600K mutations were in tumors arising in SSD skin, whereas BRAF V600E mutations were more common in tumors from sun-protected sites with low solar elastosis scores (P = 0.02).

The presence of a BRAF or NRAS mutation was associated with a lower mutation load within the tumor compared with WT tumors (P < 0.0001). This association was strongest for BRAF V600E–mutant tumors [relative risk ratio (RRR) = 0.38/mutation; 95% confidence interval (CI), 0.16–0.91], and less strong for BRAF V600K- and NRAS-mutant tumors (RRR = 0.91/mutation; 95% CI, 0.82–1.02 and RRR = 0.82/mutation; 95% CI, 0.69–0.97, respectively).

This cohort of melanomas exhibited a range of nonsynonymous mutations in genes classically associated with melanoma including c-KIT (n = 3), RAC1 (n = 2), and CDKN2A (n = 3). Other melanoma-associated mutations validated in the literature as occurring in more than 10% of cases are shown in Fig. 1. The majority of these mutations clustered in tumors with high mutation loads.
BRAF/NRAS WT tumors

Ten patients in this study had tumors lacking canonical BRAF and NRAS mutations. The median age was 78 years compared with 66 years in the BRAF/NRAS–mutant group, although this difference was not statistically significant. BRAF/NRAS WT tumors, compared with tumors carrying NRAS or BRAF mutations, were more common on the head and neck (P = 0.04) and from SSD skin (P = 0.01). This is in keeping with previous observations in BRAF-mutant tumors (33). BRAF/NRAS WT tumors had a higher average mutation rate (27 vs. 5.6 mutations/Mb; P = 0.0001).

Previous studies have shown that BRAF-mutant melanomas are commonly of SSM subtype and typically display characteristic histopathologic features including epidermal thickening and peripheral circumscription. In our study, six BRAF/NRAS WT tumors were SSM, three were NM, and one was LMM subtype. The contour of the epidermis within the radial growth phase of BRAF/NRAS WT tumors was thinner compared with the adjacent epidermis, with a discontinuous transition from tumor to normal skin compared with a more abrupt cutoff in mutant tumors (P = 0.02). There was no statistically significant association between BRAF/NRAS WT tumors and other histopathologic features (tumor thickness, mitotic rate, ulceration, regression, pigmentation, tumor-infiltrating lymphocytes, cell size, or predominant cell type).

Most of the BRAF/NRAS WT tumors harbored a number of recently validated melanoma gene mutations (Fig. 1). From our filtered list of mutations, those occurring in genes reported in the COSMIC database were identified. The average number of COSMIC mutations was 48 in the BRAF/NRAS WT group compared with 12 in the BRAF and NRAS groups (P = 0.0004; Fig. 5A). Although COSMIC details mutations which may or may not be actionable, GDSC database contains an enriched set of 70 genes, which have drug-sensitivity information linked to mutational profiles of cancer cells. The average number of potentially actionable mutations in genes reported in the GDSC database was 11 in the BRAF/NRAS WT group compared with three in BRAF and four in NRAS groups (P = 0.0003; Fig. 5A).

Examination of somatic mutations in these tumors indicated that a majority had actionable mutations in known cancer genes as shown in Supplementary Table S4. All potentially actionable mutations were independently confirmed by Sanger sequencing. Five tumors had mutations in
the tumor suppressor gene \textit{NF1}. \textit{KIT} mutations were present in three tumors, all with SSD skin ($P = 0.05$) and a higher than average mutation rate of 30 per Mb ($P = 0.02$). Actionable hotspot mutations in \textit{RAC1} (P29S; refs. 14, 15), \textit{PDGFR\(\alpha\)} (E996K; ref. 34), and \textit{HRAS} (G13D; ref. 35) were also identified in individual tumors. In addition, the patient with a somatic \textit{HRAS} mutation also had a point mutation in the \textit{KIT} gene together with a truncating mutation in \textit{CDKN2A} (Supplementary Table S4).

Three tumors in the \textit{BRAF/NRAS} WT group had fewer than 10 mutations per Mb. Interestingly, two of these tumors had mutations in \textit{NOTCH1}, one of which had a concurrent \textit{TP53} mutation. Other potentially actionable mutations in this group are shown in Supplementary Table S4.

**Validation of high mutation load in \textit{BRAF/NRAS} WT tumors**

Of 198 cutaneous melanomas (42 primary and 156 metastatic samples, excluding acral, uveal, mucosal, and unknown primary melanomas) sequenced by Hodis and colleagues (14) and Krauthammer and colleagues (15), 46% were \textit{BRAF} V600E–mutant, 9% were \textit{BRAF} V600K, 23% were \textit{NRAS}-mutant, and 21% were \textit{BRAF/NRAS} WT.
elastosis scores and increasing mutation rate (P) compared with described melanoma pathways in tumors, respectively (BRAF 495, and 940 for The mean number of somatic substitutions was 397, 577, and 940 for BRAF V600E, V600K, NRAS, and WT tumors, respectively (P = 0.006).

Pathway analysis
An analysis of the number of mutations across eight well-described melanoma pathways in BRAF- and NRAS-mutant tumors compared with BRAF/NRAS WT tumors is shown in Fig. 5B and C. Although the MAPK pathway contains the majority of mutations for all groups, BRAF/NRAS WT tumors have multiple mutations spread across seven of these pathways, in particular the PI3K-Akt and p53 pathways. NRAS mutants on the other hand, had a slight preponderance to other pathways but NRAS remains the dominantly mutated gene.

Discussion
This study shows that BRAF and NRAS WT melanomas are a complex group with a high mutation load due to extensive UV damage. This finding was validated in a larger cohort of 198 primary and metastatic melanomas. BRAF/ NRAS WT melanomas are strongly associated with UV damage as evidenced clinically by the higher degree of solar elastosis, and on a molecular level, with a high proportion of C>T transitions at a dipyrimidine, and more specifically, more frequent tandem C>T transitions. It is likely that different treatment strategies will be required when treating patients with high mutation load melanomas, which harbor an array of potentially targetable mutations. Classification of melanoma into BRAF-mutant, NRAS-mutant, and high-mutation load groups may be helpful for identification of patients suitable for particular combined drug therapies.

To our knowledge, this is the first study to sequence primary cutaneous melanomas and correlate molecular data with clinical characteristics. Although previous studies have shown an association between molecular signatures of UV damage and cutaneous melanomas (14, 15), none have quantified the degree of solar elastosis adjacent to the primary tumor. In fact, melanomas arising in nonglabrous, nonmucosal sites are often universally classified as “sun-exposed.” Kruthammer and colleagues also found that BRAF/NRAS WT cutaneous melanomas had a high mutation burden, but reported BRAF/NRAS–mutant melanomas to have mutation loads in the mid range. That cohort was skewed by uveal, acral, and mucosal tumors with low mutation burdens. Although acral melanomas can have BRAF or NRAS mutations and perhaps be associated with UV damage (36), mucosal [which may be NRAS- or BRAF-mutant (14, 15, 37)], and uveal melanomas are not UV related. With histologic assessment of solar damage, we have been able to show distinct molecular signatures among common histologic subtypes of cutaneous melanoma, which may have important implications for treatment.

The inverse correlation between mutation load and primary tumor thickness shown in this study was surprising. The clonal evolution model rests on the notion that cancer progresses from a low metastatic potential (thin early-stage melanomas) to a strong metastatic state (thicker more locally advanced melanomas) through the accumulation of molecular alterations such as mutations which increase the invasive and proliferative potential of cancer cells as tumor burden increases (38). Our data suggest that in melanomas there is not a simple relationship between tumor burden and mutation load. Rather, it suggests that melanomas do not necessarily accumulate mutations as they get thicker. In support of this, a similar range of mutation rates published for metastatic disease and cell
The median thickness of primary melanomas in this cohort (6.2 mm) is considerably thicker than the median thickness of melanomas at the time of diagnosis for the general population. In Victoria, Australia, the median thickness of melanomas at the time of diagnosis for the general population is 0.6 and 2.6 mm, respectively (40). Females were over-represented in this cohort as compared with the general melanoma population. This ascertainment bias relative to the general population is due to the difficulties associated with acquiring fresh tissue samples from primary melanomas. Tumors need to be thick to take a fresh sample for tumor banking while ensuring adequate material is available for routine pathologic assessment and sufficient tumor material remains for DNA extraction and sequencing (40, 42).

Given all primary tumors were thick (>2 mm) and numbers in this cohort were small, it is not possible to conclude that BRAF-mutant tumors are thicker in general. In fact, larger studies have shown that the frequency of BRAF V600E mutations decreases with tumor thickness (1, 43). Our findings are in agreement with other studies, which show that BRAF V600E mutations are more prevalent in non-SSD melanomas. Tumors need to be thick to take a fresh sample for tumor banking while ensuring adequate material is available for routine pathologic assessment and sufficient tumor material remains for DNA extraction and sequencing (40, 42).
skin (1), and V600K and KIT mutations are more prevalent in SSD skin (6, 44). Furthermore, our findings that BRAF and NRAS WT tumors tend to have a thinner epidermis in contrast to adjacent normal skin and a tendency for the transition from tumor to normal skin to be discontinuous are also supported by larger studies (3).

The correlation between BRAF status and mutation rate suggests that where a predominant driver mutation is present, the mutation rate will often remain low. Although KIT mutations occurred in three BRAF/NRAS WT tumors, these all had a high mutation burden. There were three tumors in the BRAF/NRAS WT group with mutation rates less than 10 per Mb. Two of these tumors had a mutation in NOTCH1, which has recently been implicated in growth and invasion of uveal melanoma (45) and is potentially targetable (46, 47).

Inactivating mutations in the tumor suppressor gene NF1 were present in 50% of WT tumors compared with just 4% of BRAF/NRAS–mutant tumors. Hodis and colleagues reported a similarly high frequency of NF1 mutations among BRAF/NRAS WT compared with mutant tumors (25% vs. 2%; ref. 14). Loss of NF1 tumor suppression can lead to constitutive MAPK pathway activation through Ras (ref. 48; Fig. 5B). This highlights the potential for NF1 to become an important therapeutic target in WT tumors.

It is important to note that, given the current knowledge on the sensitivity and resistance of therapeutics to specific mutations, classifying mutations into driver and passenger mutations as well as actionable and/or druggable is difficult. An “actionable mutation” as defined earlier, is a genetic alteration which may have significant diagnostic, prognostic, or therapeutic implications for a patient. A subset of these may be “druggable,” i.e., it would predict sensitivity or resistance to a specific drug (49). Therapeutic targeting of mutations in tumor suppressor genes is particularly challenging, as research into restoring normal gene function in patients (i.e., gene therapy) is currently ineffective for most tumor suppressor genes.

Although systematic identification of actionable mutations in this cohort is particularly challenging due to the large number of mutations present, we have attempted to identify actionable mutations by comparing variants with a number of cancer mutation databases. BRAF/NRAS WT tumors contain a number of low-frequency driver mutations and therefore require a larger number of UV-induced insults to the genome to progress. Importantly, they do contain a number of potentially targetable mutations, though these are spread over different pathways. Like BRAF–mutant tumors, the MAPK pathway is most frequently involved in BRAF/NRAS WT tumors, however, this is accompanied by greater burden of mutations in this group overall. Although there is significant cross-talk between pathways, results from this study suggest that therapeutic targeting of multiple pathways may be necessary rather than focused targeting of a single pathway in BRAF/NRAS WT tumors with a high mutation burden.

Disclosure of Potential Conflicts of Interest

J.F. Thompson has other commercial research support from GlaxoSmithKline and Provacetus, has honoraria from Speakers Bureau of GlaxoSmithKline, and is a consultant/advisory board member of GlaxoSmithKline. G.A. McArthur has a commercial research grant from Novartis, Millennium, and Pfizer and is a consultant/advisory board member of Roche Genentech, GlaxoSmithKline, Novartis, Millennium, Amgen, Ventana, and Bristol-Myers Squibb. No potential conflicts of interest were disclosed by the other authors.

Authors’ Contributions


Development of methodology: V. Mar, S.Q. Wong, J. Li, R.A. Scolyer, R. Tothill, A. Dobrovic

Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): V. Mar, S.Q. Wong, R.A. Scolyer, C. McLean, G.J. Mann, J.F. Thompson, J. Cebon, J.W. Kelly, A. Dobrovic, G.A. McArthur


Writing, review, and/or revision of the manuscript: V. Mar, S.Q. Wong, J. Li, R.A. Scolyer, A.T. Papenfuss, H. Kakavand, G.J. Mann, A. Behren, J. Cebon, R. Wolfe, J.W. Kelly, A. Dobrovic, G.A. McArthur

Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): S.Q. Wong, J. Li, H. Kakavand, A. Behren, J. Cebon

Study supervision: S.Q. Wong, R.A. Scolyer, A.T. Papenfuss, G.J. Mann, G.A. McArthur

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