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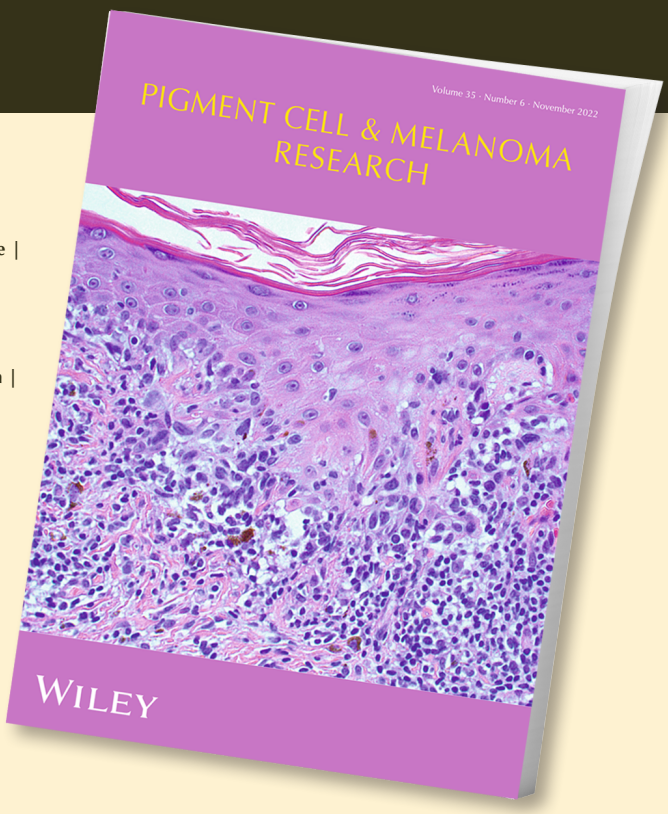

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SHORT COMMUNICATION

WILEY

Landscape of mutations in early stage primary cutaneous melanoma: An InterMEL study

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Abstract

It is unclear why some melanomas aggressively metastasize while others remain indolent. Available studies employing multi-omic profiling of melanomas are based on large primary or metastatic tumors. We examine the genomic landscape of early-stage melanomas diagnosed prior to the modern era of immunological treatments. Untreated cases with Stage II/III cutaneous melanoma were identified from institutions throughout the United States, Australia and Spain. FFPE tumor sections were profiled for mutation, methylation and microRNAs. Preliminary results from mutation profiling and clinical pathologic correlates show the distribution of four driver mutation sub-types: 31% BRAF; 18% NRAS; 21% NF1; 26% Triple Wild Type. BRAF mutant tumors had younger age at diagnosis, more associated nevi, more tumor infiltrating lymphocytes, and fewer thick tumors although at generally more advanced stage. NF1 mutant tumors were frequent on the head/neck in older patients with severe solar elastosis, thicker tumors but in earlier stages. Triple Wild Type tumors were predominantly male, frequently on the leg, with more perineural invasion. Mutations in *TERT*, *TP53*, *CDKN2A* and *ARID2* were observed often, with *TP53* mutations occurring particularly frequently in the NF1 sub-type. The InterMEL study will provide the most extensive multi-omic profiling of early-stage melanoma to date. Initial results demonstrate a nuanced understanding of the mutational and clinicopathological landscape of these early-stage tumors.

KEYWORDS
multi-omic profiling, primary melanoma, prognostic models, tumor mutations

1 | INTRODUCTION

Patients with metastatic cutaneous melanoma have greatly benefited from the development of immunotherapies and targeted therapies. However, to date, the understanding of the natural history of melanoma as it develops is incomplete. Five-year survival rates, estimated to range from 94% for Stage IIA to 82% for Stage IIC and 93% for Stage IIIA to 32% for Stage IIID (Gershenwald et al., 2017), reflect the variation between and within stages, composed of both aggressive, metastasizing tumor behavior and indolent, local growth patterns. Identifying which tumors are aggressive and which are slow to evolve is a high research priority. Pembrolizumab was recently approved for adjuvant treatment for Stage IIB and IIC melanomas, with only a small proportion of patients experiencing benefits and an equal proportion experiencing serious or permanent adverse events (Luke et al., 2020). Thus, there is great clinical need for deep exploration of the genomic landscape of these tumors to identify accurate predictors of risk and to select the appropriate patients for systemic treatment.

Since its inception more than a decade ago, The Cancer Genome Atlas (TCGA) has been the benchmark resource for investigating the genomic landscape of all major cancers (Ellrott et al., 2018). Its value is in large part due to the numbers of specimens examined and the broad range of 'omics platforms evaluated. However, primary melanoma is not well represented in TCGA, the preponderance of the samples used being metastatic. A major reason is that approximately 70% (Criscione & Weinstock, 2010) of incident melanomas are very small, containing small amounts of tumor tissue, and thus limited amounts of relevant fresh frozen genomic material such as DNA can be extracted, greatly reducing the extensiveness of the genomic analyses that were possible in TCGA. Since the goal of TCGA was to perform extensive genomic analyses across multiple platforms, the tumors used were primarily derived from fresh frozen metastatic samples (364 of the 468 cases analyzed). A further major drawback to understanding the natural history of melanoma from TCGA data is the fact that the 104 primaries included were very large with a median Breslow thickness of 10.0 mm (IQR 5–14). Other large series presenting sequencing results also included few primaries. For example, a series of 556 cutaneous melanomas (Shoushtari et al., 2021) were sequenced using a targeted next-generation sequencing (NGS) panel; however, only 104 (15%) were primaries. Other series have had even fewer primary tumors (Hayward et al., 2017). In short, while there are multiple studies looking at clinical/pathological or specific mutational status, there is a lack of multi-omic profiling studies of primary early-stage melanomas.

For these reasons, our group created the InterMEL collaboration with the goal of purposefully investigating the genomic landscape of clinically localized primary melanomas. The goal was to create an investigative resource directly applicable to this specific range of disease stages and consequently employs a breadth of genomic analyses that are feasible for the small tumors that are commonplace at earlier stages. While it was not technically feasible to assemble a truly population-based resource, our sampling was designed nonetheless to be as representative as possible of stage IIA-IIID primary melanomas while at the same time optimizing statistical power for addressing the primary goal of the study – to identify markers of survival. We thus sampled retrospectively approximately equal numbers of cases who died of melanoma within 5 years and controls who survived disease-free beyond 5 years. The cases were sampled from various hospitals or treatment centers in the USA, Australia and Spain based on the availability of tumor tissue in the pathology archives. Our genotyping plans involved targeted panel sequencing, DNA methylation profiling, and microRNA profiling, with the goal of creating integrated clinical prediction rules and understanding the biological relationships underpinning observed survival differences.

In this article, we report details of our study design and a preliminary report on the mutational profiles of the tumors sequenced to date, providing some basic descriptions of the mutational landscape observed and contrasting the results with corresponding findings from TCGA.

Significance

Although immunotherapy and targeted therapies have provided improvement in survival for many late stage melanoma patients, many earlier stage patients are now receiving adjuvant therapy, most of whom would never have progressed. This study aims to characterize in fine detail who might benefit and who might not need adjuvant therapy among Stage II and III melanoma patients. We have conducted a multi-omics study using FFPE tissue to identify aggressive and indolent melanomas and anticipate that this knowledge obtained at diagnosis will help clinicians guide patients in their choice of therapy.

2 | METHODS

2.1 | Sampling

We used a case–control design to optimize the statistical power for addressing the primary goal of the InterMEL Study, to identify genomic predictors of survival from melanoma. "Cases" were those who died of melanoma within 5 years of diagnosis (median follow up 724 days, IQR 421–1098) and "controls" were those who lived more than 5 years without progression (median follow up 3162 days, IQR 2373–4086). Each institution ($n = 15$) sought to include approximately equal numbers of patients with stage II tumors in each group (cases or controls) and equal numbers of patients with stage III tumors in each group. Each center obtained institutional review approval.

2.2 | Tissue eligibility

Eligible tumors included primary cutaneous melanomas diagnosed on or after January 1, 1998, and prior to January 1, 2016, within stages IIA-IIID, re-staged according to the AJCC 8th edition (Gershenwald et al., 2017). Tumors thus were at least 1.05 mm thick with sufficient tissue available to ensure the number of slides necessary for nucleic acid extraction (7–10 μm sections [unstained, uncharged]; two 4–5 μm sections [unstained, charged]; two H&E's, one at the beginning of the sectioning and one at the end of the sectioning). These tumors were required to be first primary invasive melanomas (no previous invasive melanomas). The patient must not have received adjuvant immunotherapy or targeted therapy prior to progression. Nucleic acid was extracted in the form of DNA and RNA for three distinct 'omics panels: mutation, methylation and miRNA profiling. Here, we describe the mutational profiles of the 518 tumors identified and analyzed to date.

2.3 | Logistics

Eligible samples were identified from 15 contributing institutions. Eligibility characteristics were verified by an investigator on the

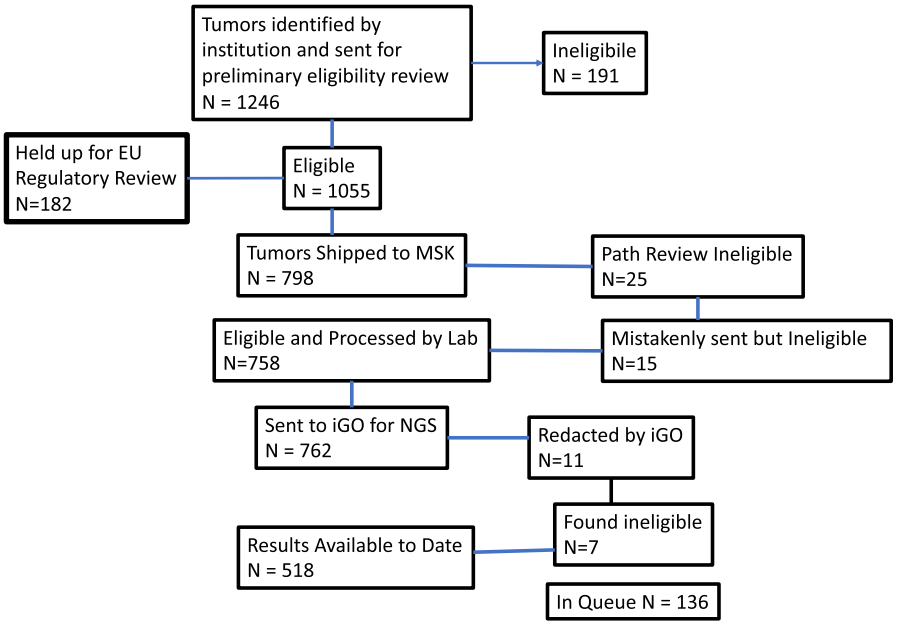

FIGURE 1 Flow of data

team (TL) and then shared with our biospecimen and pathology core which sent 2D labels to the contributing institutions for shipment. After receipt of the tissue samples, two pathologists (KB and CL) evaluated whether indeed the tissue was melanoma, evaluated cellularity and areas for molecular sampling were marked. DNA and RNA were co-extracted using the AllPrep® DNA/RNA FFPE Kit (Qiagen) and portions sent for testing with three distinct genomic platforms: mutation profiling; methylation profiling; microRNA profiling. Hemoxylin and eosin-stained slides were then sent back to the pathologists for thorough pathological review, evaluating cellularity a second time. Quality control and other technical details are discussed in Orlow et al. (2022).

2.4 | Genomic analyses

In this article, we report results solely from mutational profiling. Due to the small size of primary melanoma tumors, we used the targeted panel created at Memorial Sloan Kettering Cancer Center (MSK-IMPACT™) (Cheng et al., 2015). MSK-IMPACT™ is an FDA-approved hybridization capture-based NGS panel capable of detecting all protein-coding mutations, copy number alterations, and selected promoter mutations and structural rearrangements in 468 cancer-associated genes (Table S1). For this project, we expanded the panel to include the melanocortin 1 receptor gene (*MC1R*). MSK-IMPACT requires normal tissue samples as a benchmark. Normal DNA was obtained from a variety of sources, including blood, saliva, lymph nodes and adjacent skin. However, for 95 of the 518 tumors profiled to date, adequate normal DNA was unavailable. For these cases, we developed a robust alternative pipeline that demonstrates high accuracy for somatic mutation calls on the panel (Shen et al., 2022). The pipeline takes advantage of the allelic imbalances caused by tumor impurity to distinguish true somatic mutations from germline mutations. Briefly, this method works by examining the observed allele frequency. If the variant is truly germ-line this should be in the region of 50%, regardless of tumor purity. However, due to the fact that tumor purity is typically considerably less than 100%, it is relatively easy to distinguish somatic variants, where the allele frequency will be considerably less than 50%, from germ line variants. We were not especially concerned about mis-diagnosing important germ-line variants as somatic, such as *CDKN2A* germ-line mutations, since germ-line mutations in this gene are so infrequent, occurring in only about 1% of melanoma cases in the general population (Aoude et al., 2015; Berwick et al., 2006). Quality control was applied at every step: eligibility, nucleic acid extraction, sequencing, and post-sequencing, as well as analysis (Orlow et al., 2022).

2.5 | Definition of sub-types

We use five melanoma sub-types in our report: BRAF – mutations occurring at the category *BRAF_V600* hotspot; NRAS – mutations occurring at the *NRAS*_Q61 hotspot; NF1 – any mutation occurring on the *NF1* gene; Triple Wild Type (TWT) – tumors without any mutations on *BRAF*, *NRAS* or *NF1*; other – patients with mutations on *BRAF* or *NRAS* at locations other than V600 or Q61.

2.6 | Statistical analyses

Standard descriptive statistics were used to summarize the patient demographic and clinicopathologic characteristics. Categorical variables were summarized using counts and percentages and analyzed using chi-square statistics. Continuous variables were summarized using median and inter-quartile range (IQR). We generated oncoplots using the R Bioconductor "maftools" package (Mayakonda

et al., 2018) to describe the mutation profiles for the previously reported melanoma driver genes. The top 10 mutated genes were considered "driver" genes (Cancer Genome Atlas Network, 2015; Hayward et al., 2017; Hodis et al., 2012). We defined tumor mutational burden (TMB) for a sample to be the number of nonsynonymous mutations per megabase observed on genes on the MSK-IMPACT panel. TCGA TMB was calculated as the total number of nonsynonymous mutations divided by the length of exome sequenced (44 mb) (Zehir et al., 2017).

3 | RESULTS

Figure 1 provides a flowchart of recruitment to the study. A total of 1246 patients with available tumor specimens were identified as potentially eligible. Of these, eligibility was confirmed for 1055. Clinicopathological details for the available 518 tumors are provided in Table 1. Median age at diagnosis was 64 years, with 63% of the melanomas occurring in males, similar to melanoma of all stages in the USA with a median age of 65 years (Surveillance, Epidemiology, and End Results (SEER) Program, 2021) and 57% of melanomas occurring in males (American Cancer Society, 2021). There were 280 (54%) patients with stage II melanoma and 238 (46%) with stage III. Fifty-seven percent of the patients came from US institutions versus 43% from Australia. Median Breslow thickness was 4.0 mm, with tumor infiltrating lymphocytes (TILs) present in 90% of the tumors, ulceration in 60% and severe solar elastosis in 21%.

Table 1 provides further breakdown of the characteristics by molecular sub-type. A total of 31% of the patients were in the *BRAF* mutant sub-type and 18% in the *NRAS* mutant sub-type. These percentages somewhat lower than in TCGA Stages II and III (42.0%% and 25%, respectively; see bottom row of Table 1 for TCGA comparative results). In contrast 21% of InterMEL patients were in the NF1 mutant sub-type compared to 18% in TCGA, with a larger number of InterMEL subjects in the TWT sub-type, 26% compared to TCGA with 16%. Interestingly, when looking at the TMB within subtypes, there is a strong similarity between InterMEL and TCGA (see bottom of Table 1).The BRAF mutant sub-type was characterized by young age at diagnosis, associated nevi ($p = .004$), higher presence of TILs ($p = .008$), and tumors that were thinner despite being at a more advanced stage. The NF1 mutant sub-type had a predominance of tumors involving the head/neck ($p < .001$) and advanced

TABLE 1 Clinicopathologic characteristics

Characteristic	Overall	Subtype				
		BRAF	NRAS	NF1	TWT	Other[a]
	N = 518	*N* = 159 (31%)	*N* = 91 (18%)	*N* = 110 (21%)	*N* = 137 (26%)	*N* = 21 (4%)
Head/Neck	130 (25%)	33 (21%)	15 (16%)	46 (42%)	30 (22%)	6 (29%)
Trunk	143 (28%)	54 (34%)	25 (27%)	22 (20%)	34 (25%)	8 (38%)
Arms	92 (18%)	21 (13%)	18 (20%)	29 (26%)	20 (15%)	4 (19%)
Legs	151 (29%)	49 (31%)	33 (36%)	13 (12%)	53 (39%)	3 (14%)
Low mitotic index	149 (29%)	50 (31%)	19 (21%)	27 (25%)	39 (28%)	13 (62%)
Associated nevus	42 (8%)	24 (15%)	11 (12%)	2 (2%)	5 (4%)	0 (0%)
Regression present	54 (10%)	20 (13%)	8 (9%)	7 (6%)	15 (11%)	4 (19%)
Perineural invasion	87 (17%)	10 (6%)	10 (11%)	33 (31%)	32 (24%)	2 (10%)
Mean age	64 (52–75)	56 (46–69)	64 (54–75)	72 (59–78)	64 (53–75)	71 (62–76)
Male sex	324 (63%)	86 (54%)	62 (68%)	64 (58%)	92 (67%)	20 (95%)
US center	294 (57%)	101 (64%)	57 (63%)	64 (58%)	60 (44%)	12 (57%)
Thickness (median)	4.0 (2.5–6.5)	3.6 (2.3–6.0)	4.2 (2.5–6.0)	4.6 (2.9–7.2)	4.0 (2.8–6.8)	3.3 (2.5–5.0)
Stage II (vs III)	280 (54%)	59 (37%)	47 (52%)	85 (77%)	78 (57%)	11 (52%)
TILs absent	54 (10%)	8 (5%)	13 (14%)	13 (12%)	19 (14%)	1 (5%)
Ulceration present	313 (60%)	94 (59%)	60 (66%)	66 (60%)	83 (61%)	10 (48%)
Severe solar elastosis	107 (21%)	18 (11%)	17 (19%)	41 (37%)	27 (20%)	4 (19%)
Tumor mutational burden	9.3 (4.0–21.3)	7.3 (4.7–12.01)	11.3 (5.3–18.0)	36.7 (18.0–66.7)	12.0 (9.3–28.7)	4.0 (1.3–9.3)
Results from TCGA						
Tumor mutational burden	8.7 (3.5–19.7)	7.4 (3.5–14.8)	10.5 (5.8–20.4)	32.1 (14.6–49.3)	13.9 (9.0–20.3)	2.8 (1.1–9.6)
Subtype	308 (100%)	129 (42%)	78 (25%)	39 (13%)	48 (16%)	14 (5%)

[a]Other consists of non-hot spot mutations in BRAF and NRAS.

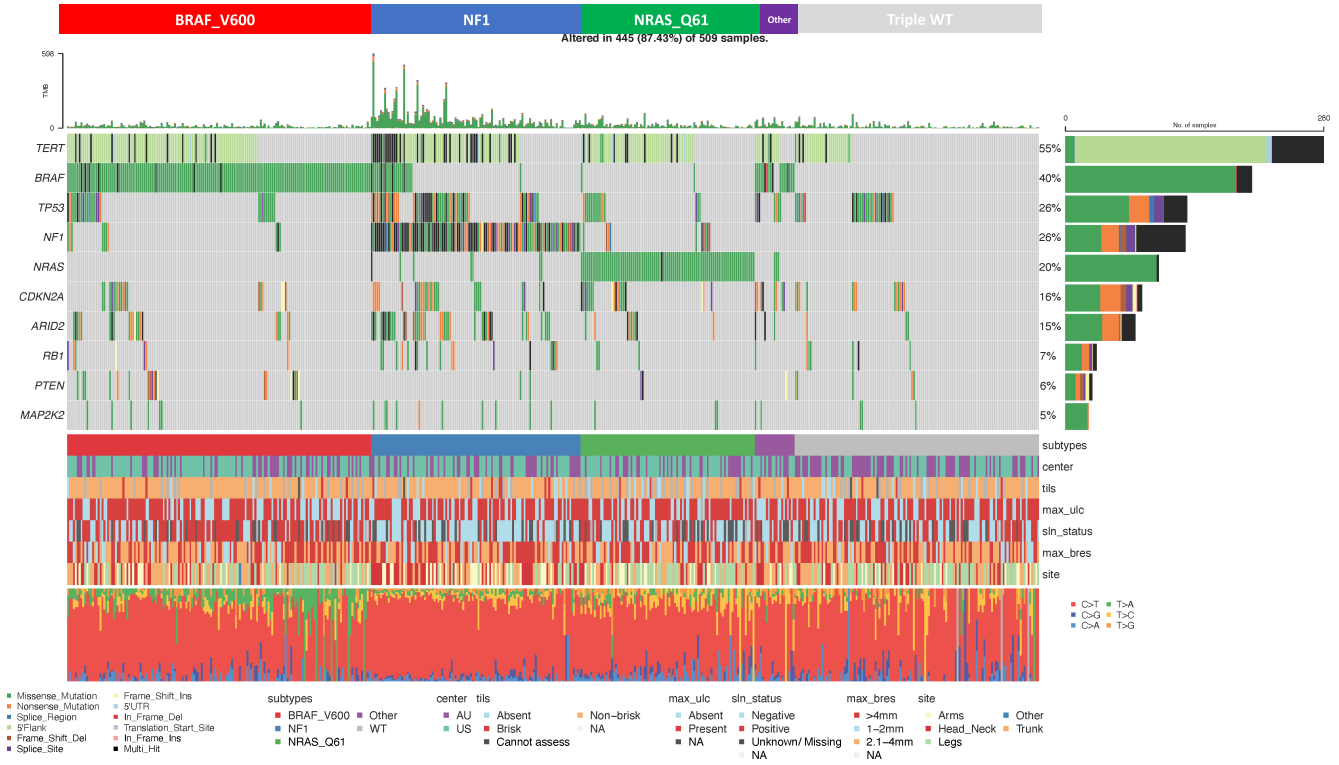

FIGURE 2 Oncoplot of melanomas, demonstrating driver mutations, sub-types, mutation type, center (US or Australia), TILs, ulceration, anatomic site, sentinel lymph node status, and Breslow thickness

age at diagnosis, more evidence of solar elastosis ($p < .001$), and earlier stage but thicker tumors ($p < .001$). The TWT sub-type occurred more often on the legs despite having a male predominance. The NRAS mutant sub-type was generally similar to the overall averages of all tumors at Stages II and III. TMB was substantially higher in the NF1 sub-type than in the other sub-types (40.0 versus 8.0, 13.3 and 6.0 for the BRAF, NRAS and TWT sub-types, respectively, $p < .001$). Further details of the mutational landscape are provided in Figure 2. Notable observations were the frequent presence of *TERT* and *TP53* somatic mutations. The *TERT* mutations appeared to be randomly distributed among the sub-types, while *TP53* mutations were concentrated in the NF1 mutant sub-type. *CDKN2A* and *ARID2* mutations were observed to occur at somewhat lower frequencies with a large proportion of *CDKN2A* mutations co-occurring with the *NF1* mutations and ARID2 generally equally dispersed among the sub-types.

4 | DISCUSSION

The fundamental goal of the InterMEL Study is to define the natural history of early-stage melanomas based on detailed information of both clinical and genomic characteristics, with a view to influencing treatment selection in a population of patients with very mixed survival expectations. The lack of clinical predictability in terms of poor outcomes, particularly in the face of systemic adjuvant therapies moving into earlier stages of melanoma, prompted us to assemble melanomas diagnosed prior to the era of immunological treatments, to understand the molecular markers predicting a natural history of shortened melanoma survival. The study of earlier stages of melanoma necessitated the use of archival FFPE tissues from generally very small tumors, an approach that was considered to be logistically and technically difficult. While the study is still in progress and results from our investigations of methylation and microRNA arrays are not yet available, the present interim report demonstrates that it is indeed possible to conduct multi-omic investigations of small tumors in a multi-center fashion. We view our study as an important complement to the influential TCGA investigation. Preliminary results from our study demonstrate generally similar genomic characteristics to TCGA, but some key differences are observed. Our results indicate that the BRAF subtype is generally less frequent in earlier stage melanomas than those represented in TCGA, with the other molecular sub-types having correspondingly higher frequencies. For example, the TWT sub-type comprises 26% of our sample versus 13% in TCGA. In most other respects, we do not find large differences between our study and TCGA.

The InterMEL study is, at its core, an effort to apply epidemiological principles to the investigation of the genomic landscape of tumors. By careful selection of archival tumor specimens and robust organization for the curation, quality control and analysis of an international, multi-center accrual of specimens, we have shown that a multi-omic study of small tumor specimens is possible and can yield a greater understanding of the molecular profiles of earlier stages of melanoma.

AUTHOR CONTRIBUTIONS

Conception and design of the study: Marianne Berwick, Nancy E. Thomas, Colin B. Begg, Ronglai Shen, Eva Hernando, Irene Orlow, Klaus Busam, Ivan Gorlov, Marc S. Erstoff, Jeffrey E. Lee and Christopher Amos. Acquisition and interpretation of the data: Li Luo, Ronglai Shen, Arshi Aurora, Irene Orlow, Marianne Berwick, Ivan P. Gorlov, Tim K. Lee. Funding acquisition: Marianne Berwick, Nancy E. Thomas, Colin B. Begg, Ronglai Shen, Eva Hernando, Irene Orlow, Klaus Busam, Tim K. Lee, Anne E. Cust, Jeffrey E. Lee, Meg Gerstenblith, Bonnie Gould Rothberg, Iman Osman and Christopher Amos. Clinical samples and data: Richard A. Scolyer, James S. Wilmott, Anne E. Cust, Graham Mann, Jennifer Ko, Pauline Funchain, Peter Ngo, Meg Gerstenblith, Cheryl L. Thompson, Judy R. Rees, Marc S. Ernstoff, Jeffrey E. Lee, Shenying Fang, Klaus J. Busam, Nancy E. Thomas, Sharon Edmiston, David Ollila, Iman Osman, Eva Hernando, Bonnie Gould Rothberg, Marcus Bosenberg, Yvonne Saenger, Paul Bogner, Charles Wiggins, Sheri Holmen, Eduardo Nagore. Writing – Original draft: Colin B. Begg, Marianne Berwick, Li Luo, Ronglai Shen, Arshi Aurora. Writing – Review and editing: Colin B. Begg, Marianne Berwick, Li Luo, Ronglai Shen, Arshi Aurora, Irene Orlow Nancy Thomas, Eva Hernando, Richard A. Scolyer, Anne E. Cust, Meg Gerstenblith, Cheryl L. Thompson, Christopher Amos, Marc S. Ernstoff, James Wilmott, Pauline Funchain, Kathleen Conway. Approval of the final submitted manuscript: All the authors.

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CONFLICT OF INTEREST

Richard A. Scolyer has received fees for professional services from F. Hoffmann-La Roche Ltd, Evaxion, Provectus Biopharmaceuticals Australia, Qbiotics, Novartis, Merck Sharp & Dohme, NeraCare, AMGEN Inc., Bristol-Myers Squibb, Myriad Genetics, GlaxoSmithKline. All others have nothing to declare.

DATA AVAILABILITY STATEMENT

The data underlying this article are subject to an embargo until 12/1/23. Once the embargo expires, the data will be available on the National Cancer Institute dbGaP website upon request.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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