Molecular subtype, biological sex and age shape melanoma tumour evolution

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Summary

Background Many cancer types display sex and age disparity in incidence and outcome. The mutational load of tumours, including melanoma, varies according to sex and age. However, there are no tools to explore systematically whether clinical variables such as age and sex determine the genomic landscape of cancer.

Objectives To establish a mathematical approach using melanoma mutational data to analyse how sex and age shape the tumour genome.

Methods We model how age-related (clock-like) somatic mutations that arise during cell division, and extrinsic (environmental ultraviolet radiation) mutations accumulate in cancer genomes.

Results Melanoma is driven primarily by cell-intrinsic age-related mutations and extrinsic ultraviolet radiation-induced mutations, and we show that these mutation types differ in magnitude and chronology and by sex in the distinct molecular melanoma subtypes. Our model confirms that age and sex are determinants of cellular mutation rate, shaping the final mutation composition. We show mathematically for the first time how, similarly to noncancer tissues, melanoma genomes reflect a decline in cell division during ageing. We find that clock-like mutations strongly correlate with the acquisition of ultraviolet-induced mutations, but critically, men present a higher number and rate of cell-division-linked mutations.

Conclusions These data indicate that the contribution of environmental damage to melanoma likely extends beyond genetic damage to affect cell division. Sex and age determine the final mutational composition of melanoma.

What is already known about this topic?

- Cancer incidence and mortality are influenced by sex and age.
- Melanoma is more frequent in men, and the incidence and mortality rise with increasing age.
- The main mutations driving melanoma are linked predominantly to ultraviolet (UV) radiation damage and to errors accumulated in the DNA after each cell division, which are un repaired.
- These clock-like mutations linked to cell division accumulate steadily over time in both healthy tissue and cancers.

What does this study add?

- Clock and UV mutations are tightly correlated and arise in melanoma as a function of age and sex.
- The molecular subtypes have a distinct pattern and rate of UV and clock mutations, and clock mutations depend on the amount of UV damage.
Sex and age disparity in cancer incidence and outcomes are well described, and studies have revealed age\(^1,2\) and sex differences\(^3\) in genomics.\(^4,5\) Somatic mutations arise in cells due to damage following cell-intrinsic processes, as well as due to external environmental damage on the DNA. Recent work describes computational methods to discern the multiple, distinct signatures of DNA damage imprinted on DNA depending on the insult,\(^6\) but to date there are no available models to study the relationship between the distinct damaging processes.

Cutaneous melanoma exemplifies a cancer type primarily presenting cell-intrinsic (cell division) and environmental (ultraviolet radiation, UVR) damaging processes,\(^7\) as well as presenting an age and sex bias. Male patients and the aged population have a higher incidence and rate of death, so we studied whether the genomic imprints of the major contributors to total autosomal tumour mutation burden (TMB) in melanoma are possible sources for the disparity.

Melanoma presents a broad range of clinical subtypes, categorized by age of onset, history and pattern of UVR exposure.\(^8,9\) At one end of the spectrum, we identify elderly patients with melanomas arising at anatomical sites that have been chronically exposed to UVR, who have a high TMB. In contrast, melanoma in younger patients arises decades after sunburn, over skin that is intermittently exposed to UVR, with a lower TMB.\(^9\)–\(^11\)

The mutually exclusive oncogenic drivers \(^{V600}BRAF\) and \(^{NRAS}\) underpin the majority of cutaneous melanomas.\(^12\) Loss-of-function mutations in the tumour suppressor neurofibromin (NF)1 drive an additional subset of cases, and a further subgroup is defined by the absence of \(^{V600}BRAF\), \(^{NRAS}\) and \(^{NF1}\) mutations (triple wildtype, W3).\(^13\) These genetically distinct categories overlap to some extent in their clinical characteristics, with \(^{V600}BRAF\) being more prevalent in younger patients.\(^12\)

Here we examine the relationship between mutational processes and their contribution to the melanoma somatic mutation load, and their variation over time and across sexes. We provide a mathematical framework to model how the specific damage patterns in DNA arise over time and across the sexes. Analysing the strong bias in the mutational landscape could point to key biological differences in how tumours develop and evolve during ageing and across sexes.

**Materials and methods**

**Mutation data**

The primary data are the somatic mutation calls from The Cancer Genome Atlas (TCGA) Mutation Annotation Format of the whole-exome sequences of the Skin Cutaneous Melanoma (SKCM) cohort.\(^10\) The sequencing data were obtained from the TCGA in accordance with ethical guidelines.

We classified samples by their mutations in \(^{BRAF}\) with \(^{V600}\) mutations, \(^{NRAS}\), \(^{NF1}\) or none of these genes. Samples with \(^{V600}BRAF\) or \(^{NRAS}\) and an additional \(^{NF1}\) mutation were classified as either \(^{V600}BRAF\) or \(^{NRAS}\). We inferred the mutational processes by categorizing the single-nucleotide substitutions in the trinucleotide context and used mathematical models to infer their contribution to the mutational landscape across biological sex and age.

We estimated the exposure to Signatures 1 and 7 using the R package deconstructSigs\(^14\) and validated the approach by rederiving the mutation signatures using a hierarchical Dirichlet process (R package hdp; R Foundation for Statistical Computing, Vienna, Austria).

**Mathematical models**

The number of mutations present at any given age can be described using a Poisson process\(^15\) with time-varying mean \(\lambda(t)\).\(^15\) We use an exponential model \(\lambda(t) = N_0 e^{at}\) for the mean. To estimate the effect of the different subtypes on the ratio of mutations by age we modelled the accumulation of mutations using a homogeneous Poisson process with age as an offset:

\[
\log E[N(t)|X_1, \ldots, X_i] = \alpha + \sum_{i} \beta X_i,
\]

where the \(X_i\) values are the covariates (sex, site and subtype in our case). As the distribution of the mutation count data was
found to be overdispersed (R package aes), we estimated a negative binomial regression instead of a Poisson regression. By fitting a Poisson mixture model, we found similar ratios between the coefficients associated with the different subtypes. We estimated the change in accumulation rate with age with the exponential model for mutation accumulation:

\[ N(t) = N_0 e^{\alpha t}; \]
\[ \text{the derivative of } f(t) = e^{\alpha t} \text{ by } t \text{ is } \frac{df}{dt} = \frac{e^{\alpha t}}{t} (\alpha - 1). \]

Using the exponential accumulation model, \[ N(t) = EN_0 e^{\alpha t}, \]
where \( E \) (for extrinsic mutations) denotes the number of Signature 7 mutations (Poisson regression with the logarithm of Signature 7 as offset), we estimated the ratio \( N(t)/E \) of intrinsic, clock-like mutations when factoring out the extrinsic, Signature 7 mutations.

Additional methods are described in Appendix S1 (see Supporting Information), including mutation data, mutation signatures, the mathematical model of mutation accumulation, change in accumulation rate with age and cell-cycle gene expression analysis.

**Results**

**Clock-like and ultraviolet radiation-driven mutations accumulate with age at distinct rates in the molecular subtypes of melanoma**

We catalogued the base substitutions in 396 whole-exome cutaneous melanoma samples from TCGA (TCGA-SKCM)\(^{12}\) according to 96 categories defined by the base substitution and the preceding and following bases.\(^7\) In total, 172 had a \( \text{V}_{600E}\text{BRAF} \) mutation, 96 \( \text{NRAS} \) and 44 \( \text{NF1} \), and 84 samples were non-\( \text{BRAF} \), non-\( \text{NRAS} \) and non-\( \text{NF1} \) (\( \text{W3} \); Table S1; see Supporting Information).

We inferred the mutational signatures that account for the somatic mutations from the TCGA data. We extracted the DNA mutational signature linked to UVR (Signature 7, COSMIC database), which is present to varying degrees across melanomas. Next, we identified the intrinsic, age-related signature observed in normal cells and cancers with high cell turnover, which corresponds to spontaneous deamination of methylated cytosine residues into thymine at CpG sites that remain unrepaired due to rapid DNA replication (Signature 1, COSMIC database).\(^{7,16,17}\) This clock-like mutational process allows estimation of the number of divisions a cell has undergone since its inception. Previous studies have modelled the potential disruption to the linear acquisition of somatic mutations during ageing that occurs when the neoplastic phase alters the rate of mutation acquisition, and found across multiple cancer types that clock-driven mutations are linked to intrinsic cellular division despite neoplastic and oncogenic driver ontogenesis.\(^{16,18}\)

We confirmed a positive correlation between the median number of Signature 1 mutations per year and age for all samples (Spearman \( \rho = 0.41, P < 0.001 \); Figure 1a), indicating that these mutations accumulate with age. \( \text{NRAS} \), \( \text{NF1} \) and \( \text{W3} \) melanomas presented an increase in the mean Signature 1 mutations with age, but this relationship was less significant in \( \text{NF1} \) and \( \text{W3} \) melanomas, likely due to the lower sample size (\( \text{NRAS} \) Spearman \( \rho = 0.35, P < 0.01 \); Figure 1b). Strikingly, there was no significant rank correlation between Signature 1 and age in \( \text{BRAF} \) samples (Spearman \( \rho = 0.03, P = 0.41 \)). To examine the difference in the rates of Signature 1 mutation accumulation between \( \text{BRAF} \), \( \text{NRAS} \), \( \text{NF1} \) and \( \text{W3} \) melanomas, we determined the ratio between the number of Signature 1 mutations and age, and found significant differences in the ratios of \( \text{BRAF} \) and \( \text{W3} \) to \( \text{NF1} \) melanomas (pairwise Wilcoxon rank sum test with Bonferroni correction, \( P < 0.0027 \); Figure 1c), but these were less pronounced for \( \text{BRAF} \) and \( \text{NRAS} \) samples.

Next we examined the relationship between Signature 1 mutations and melanoma cell proliferation by investigating the gene expression of cell-cycle genes.\(^{19}\) We found a weak but significant correlation between Signature 1 and both cell-cycle checkpoint \( \text{G}_1/\text{S} \) (\( P = 0.03, R = 0.107 \)) and \( \text{G}_2/\text{M} \) (\( P = 0.016, R = 0.121 \)) expression genes. Furthermore, when dividing the melanomas by high vs. low Signature 1 mutations based on the median, we observed a significantly higher expression of both \( \text{G}_1/\text{S} \) and \( \text{G}_2/\text{M} \) genes in the high Signature 1 group, which was more significant for the \( \text{G}_2/\text{M} \) expressed genes. Finally, in a multiple regression with the other factors in the dataset (age, sex and molecular subtype), Signature 1 was the only factor associated with \( \text{G}_1/\text{S} \) and \( \text{G}_2/\text{M} \) gene expression.

We next examined the contribution of Signature 7 mutations and found a progressive increase as patients aged, in accordance with progressive accumulation of UVR damage during the course of life (Spearman \( \rho = 0.37, P < 0.006 \); Figure 1d). However, the rate of mutations varied depending on the molecular subtype, with no significant correlation found in \( \text{BRAF} \) melanomas between Signature 7 and increasing age (Spearman \( \rho = -0.15, P = 0.87 \); Figure 1e), and \( \text{NRAS} \) samples showing a steady increase of Signature 7 with age (Spearman \( \rho = 0.37, P < 0.006 \)). Similarly to Signature 1, there was a significant difference in the ratio of Signature 7 mutations to age across the subtypes (\( P < 0.03 \), Mann–Whitney U-test with Bonferroni correction; Figure 1f).

We investigated the association between Signature 7 (global UV damage) and the novel probabilistic UV damage signatures that were more recently defined.\(^{13}\) We calculated the likely UV-associated mutational signatures with the version 3 signatures, using deconstructSigs for consistency. The correlation between the sum of the four new signatures (\( \text{SBS7a}, \text{SBS7b}, \text{SBS7c}, \text{SBS7d} \)) and the version 2 Signature 7 is \( > 0.95 \) (\( P < 0.001 \)). The vast majority of mutations contributing to these signatures are SBS7a (median proportion of total Signature 7 per sample = 0.474) and SBS7b (median proportion per sample = 0.495). As both signature mutagens encompass the canonical \( \text{CC} \rightarrow \text{TT} \) and the atypical frequency of \( \text{C} \rightarrow \text{T} \) substitutions at a dipyrimidine site that is attributable to UV
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mutagenesis, we retained the original, comprehensive Signature 7 to strengthen our power to detect associations.

**Ageing affects the dynamics of the mutational landscape**

Common models of cancer have assumed that mutations accumulate at a linear rate over time. Genetic changes accumulate from early life, and a decline in replicative function with age is visible in many tissues. We used our cohorts to test the relationship between ageing and clock-like mutation rate in melanoma, and found that the ratio of mutations per year decreases with age (Spearman $\rho = -0.34$, $P < 0.005$; Figure 2b). Specifically, the decline in mutations per year is pronounced in BRAF (Spearman $\rho = -0.44$, $P < 0.001$) but not statistically significant in NRAS or W3 melanomas. We did not include NF1 samples in the analysis, as this subtype is almost exclusive to the elderly.

To analyse the differences in ageing dynamics, we considered the mean number of mutations at each age, modelled by a Poisson distribution with age-dependent rate, and found that the ratio of mutations by age decreases in older age groups (Figure 2a). We used an overdispersed Poisson (negative binomial) regression to estimate the parameters of the exponential model for each subtype BRAF, NRAS and W3, and found that overall, the amount of Signature 1 mutations increases by a multiplicative factor of $e^q = 1.012$ per year (Figure 2c). In contrast, the increase factor is only 1-005 for BRAF, 1-016 for NRAS and 1-024 for W3 melanomas (Figure 2d; and Tables S2 and S3; see Supporting Information). Thus, while the ratio of Signature 1 damage acquisition to age in BRAF and NRAS melanomas decreases during ageing, likely reflecting a deceleration of the cell proliferation rate during maturity, the ratio per year of clock mutations in W3 melanomas slightly increases during the human lifespan, reflecting a distinct behaviour (Figure 2e).

**The rate of clock-like mutations is linked to ultraviolet radiation damage**

Previous experiments showed that acute UVR drives melanocyte proliferation, but the long-lasting effects of UVR on cell division have not been explored. We investigated the proportion of Signature 1 and Signature 7 across the melanoma subtypes and found that UVR underpins approximately 75% of all mutations in BRAF, NRAS and NF1 samples, while only half of the mutations in W3 samples are accounted for by UVR (Figure 3a). Furthermore, we found a greater proportion of the ageing signature that is uncoupled from cellular division (Signature 5, characterized by T:A→C:G transitions) contributing to the overall mutational burden of W3 melanomas. The underlying biological process driving Signature 5 mutations is unknown, but it is linked to ageing independently of cellular division.

We then used Signature 1 and 7 to investigate the relationship between UVR damage and the cell cycle, and show that cell division rate, predicted from Signature 1, tightly correlates with the total UVR-induced mutations in melanoma (Spearman $\rho = 0.82$, $P < 0.001$; Figure 3b). The correlation between Signatures 1 and 7 remains significant across all of the subtypes (BRAF: Spearman $\rho = 0.70$, $P < 0.001$; NRAS: $\rho = 0.72$, $P < 0.001$; NF1: $\rho = 0.8$, $P < 0.001$; W3: $\rho = 0.64$, $P < 0.001$; Figure 3c). There is a marked difference in the increase of Signature 1 that is dependent on Signature 7 in both BRAF and NRAS melanomas (BRAF: robust regression with slope 0.033, $P < 0.001$; NRAS: robust regression with slope 0.046, $P < 0.001$). These data suggest that UVR increases the total mutation burden by damaging DNA directly, but it may also modify the TMB by affecting the dynamics of cell division. Importantly, these data show that cell proliferation is coupled to UVR, not age, in BRAF melanomas.

**Ageing-associated mutations accumulate at different rates in men and women**

Melanomas from men present an overall higher number of missense mutations than in women, adjusted for age and relevant clinical covariates. We investigated the relationship between Signature 1 and sex. Critically, we observed that men present a higher number of Signature 1 mutations per age ($P < 0.01$, Mann–Whitney U-test with Bonferroni correction; median male-to-female ratio 1:24; Figure 4a). The difference is visible in the BRAF and NRAS subtypes (Figure 4b), although it is less statistically significant ($P = 0.1$ for BRAF, $P = 0.6$ for NRAS; Mann–Whitney U-test with Bonferroni correction). For men, we found a significant rank correlation with age (Spearman $\rho = 0.45$, $P < 0.001$), but we did not find this for female samples (Spearman $\rho = 0.12$, $P = 0.35$; Figure 4a). Using multivariate negative binomial regression we found that sex affects the rate of mutation accumulation ($P < 0.001$) and estimated the factor...
Figure 2 Ageing affects the intrinsic mutation rate of the molecular subtypes. (a) Distribution curves displaying Signature 1 mutation frequency across age ranges. (b) Correlation analysis between clock-like Signature 1 mutations per year and age in cutaneous melanomas. The dots represent the median number of mutations for each age. (c) Exponential model for the accumulation of Signature 1 mutations in all melanomas. This curve models the Poisson mean distribution of mutations at each age, with age-dependent rate. (d) Exponential model for the accumulation of Signature 1 mutations in the molecular subtypes of cutaneous melanoma. This curve models the Poisson mean distribution of mutations at each age, with age-dependent rate. (e) Change in Signature 1 mutations per year with age across the BRAF, NRAS and triple-wildtype (W3) subtypes.
by which mutations increase per year in male and female samples (Figure 4c, d; and Table S3; see Supporting Information). We observed that only male samples presented a factor of mutation increase per year greater than one. The results remain robust when restricting the analysis to the molecular subtypes (except for NF1 due to the sample size), showing an increase in Signature 1 mutations with age in male samples (Table S3).

We next investigated whether the difference in the rate of mutation accumulation persists when accounting for the effect of UVR-driven Signature 7 mutations. Assuming that the number of Signature 1 mutations is proportional to the number of Signature 7 (Figure 3b, c), we investigated the ratios of Signature 1 to Signature 7 mutations per year adjusted for the effect of Signature 7 on cell division, and found that there is little to no increase in Signature 1 mutations per year in either sex. Moreover, the multiplicative rate of increase of the ratio of Signature 1 to Signature 7 mutations per year turns out to be slightly smaller (by 0.01) in men (Table S4; see Supporting Information) in all samples, across BRAF and NF1 subtypes, and is not detected in NRAS and W3. These data imply that the rate at which clock-like mutations accumulate per year depends on UVR, and this dependence is stronger in men than in women. Thus, men and women exposed to equal doses of UV adjust their cell cycles differently, with men increasing the rate of cell division.

In summary, our results indicate that the mutation rate due to cell division is determined by UVR exposure, and men are

Figure 3 The Signature 7 ultraviolet radiation (UVR) imprint predominates in melanoma and is tightly correlated to cell division Signature 1 mutations. (a) Mutation signature proportions in BRAF, NF1, NRAS and triple-wildtype (W3) cutaneous melanomas. (b, c) Correlation analysis between somatic mutations due to extrinsic, UVR-driven Signature 7 mutations in cutaneous melanomas and intrinsic, clock-like Signature 1 mutations: (b) all melanomas, (c) by melanoma subtype.
more susceptible to UVR-induced cell proliferation. In contrast, women accumulate fewer Signature 1 mutations, at a slower rate, despite UVR exposure (Figure 5).

Discussion

Sex and age differences have been observed in cancers.\cite{3,23} We provide a mathematical framework to analyse the relationship between different damaging processes shaping the mutational landscape of cancer, and how mutations can reveal the effect of age and sex on carcinogenesis. We use the predominant mutational processes of the exomes of cutaneous melanomas, which are imprinted primarily by the clock-like changes due to cell division and UVR-driven mutations. Both processes increase during ageing and are tightly correlated, which poses the intriguing possibility that UVR not only drives melanoma by damaging DNA directly, but also may influence the intrinsic processes of cell division and damage repair.

We observed that the correlation of mutations to age is absent in BRAF melanomas, in sharp contrast to NRAS and NF1 melanomas, where there is a gradual increase in UVR and cell division mutations with patient age. Although our
correlation studies do not imply causation, the punctuated rate of mutation accumulation we observe in BRAF melanomas could be driven by episodic sunburn. Our model supports clinical studies showing that BRAF melanomas are more prevalent over intermittently sun-exposed skin with little sun damage in the dermis of younger patients.\textsuperscript{24–26} Additionally, our data corroborate mouse studies demonstrating a link between few episodic UV exposures and BRAF melanoma.\textsuperscript{22,27} However, in addition to the UV-induced DNA mutation burden, UV also contributes to tumorigenesis by triggering inflammation,\textsuperscript{28} and this contribution of UV is not captured by our model.

Recent studies examining the correlation between lifetime risk of cancer and cell division have shown that tissues with higher cell turnover present an increased cancer incidence,\textsuperscript{29,30} suggesting that more proliferative tissues require less environmental damage to drive tumorigenesis. Our results challenge this assumption and propose that extrinsic processes such as UVR can modulate the contribution of cell division to mutation burden.

We show that the decline in cell division during ageing in healthy tissues and stem cells\textsuperscript{21,31} is discernible in the genomic imprint of melanoma cells. Moreover, the rate of proliferative decline is not uniform across all subtypes. Our framework could test whether the decline in cell division with age varies according to the tissue of origin, and whether decline in cell division mirrors a decrease in cancer incidence observed in the superaged population.

Finally, we reveal an increase in cell-division-linked mutations in men, which could be due to an increase in the inherent proliferation rate of male melanocytes, or to a decrease in the mutational repair of UVR mutations. A recent pan-cancer analysis has shown sex biases in mutational load, tumour evolution and mutational processes, and also at the gene level.\textsuperscript{4,5,32} Our study suggests that sex differences in melanoma cannot be explained by lifestyle or age alone, and likely reflect sex-specific biology. Intriguingly, Signature 1 is increased in women in an age-adjusted, pan-cancer analysis,\textsuperscript{32} while our results reveal a contrary sex bias in Signature 1 in melanoma.

One limitation of applying mutational signatures to infer disease evolution is that different mutational stresses occur at different timepoints of disease progression. In melanoma, UV damage is acquired when melanoma is located in the skin. In contrast, Signature 1 summarizes cell divisions throughout the lifespan of the cell, from premalignancy to advanced stages. However, recent work found most melanoma mutations to be primarily early, truncal and monoclonal.\textsuperscript{33}

Our study provides new tools to examine the rate of mutation accumulation in cancer, and to study how sex and age contribute to tumour development. Tumour burden, age and sex are known to influence the response to immunotherapies,\textsuperscript{34,35} and future studies should address how these data can be leveraged to predict response to therapy and design strategies to improve survival. Although it is limited to a single melanoma cohort, this work supports the rationale for using the mutational processes, together with age and sex, to stratify patients for novel immunotherapy trials, as well as to inform public health prevention and diagnostic campaigns.

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