Article:
Poźniak, J, Nsengimana, J, Laye, JP et al. (15 more authors) (2019) Genetic and Environmental Determinants of Immune Response to Cutaneous Melanoma. Cancer Research, 79 (10). pp. 2684-2696. ISSN 0008-5472

https://doi.org/10.1158/0008-5472.CAN-18-2864

Supplementary methods:

The Leeds Melanoma Cohort

A cohort of 2184 primary melanoma patients was recruited in the North of England in the period 2000 to 2012 (67% recruitment rate) [1][2]. Clinical data were extracted from medical records by research staff. Rigorous follow up was conducted using the National Cancer Registry, medical records in primary and secondary care and by annual questionnaires completed by consenting participants, in order to derive melanoma specific survival (MSS). We know of only 16 out of 703 patients who received checkpoint inhibitors, but we cannot exclude the possibility of an unrecognized moderating effect of these treatments. The histological features of the tumors were reported by clinical dermatopathologists and slides re-examined by a blinded single observer (Sally O'Shea). The FFPE tumor blocks were sampled horizontally through the advancing tumor edge using 0.6mm tissue microarray needles. The blocks were not sampled if the tissue was excessively necrotic or the remaining tissue in the block would be insufficient for further clinical use.

The 25-hydroxyvitamin $D_{2/3}$ levels (referred to here as vitamin D) were measured in a serum sample obtained at recruitment as previously described [1] and results adjusted for the season of blood drawing given seasonal variation.

Primary melanoma cell lines validation experiment

Melanoma cells were cultured using the selective adherence method, which was shown to limit fibroblast growth and ensured the successful generation of patient-derived 103 primary cell lines [3]. These cells were cultured in in RPMI 1640 (Sigma Life Science, USA) supplemented with 10% fetal bovine serum (Gibco, Life Technologies, USA), 2mM glutamine (Biochrom, Germany) and sodium pyruvate (Sigma Life Science, USA). One microgram of total RNA from melanoma cell cultures (103) was extracted using the Qiagen RNA mini kit (QIAGEN, USA) according to the manufacturer's protocol. RNA capture was performed with the TruSeq RNA Library Prep Kit v2 (Illumina,USA) and the library was sequenced on a HiSeq4000 (Illumina). RNA counts were quantified from single-end reads using STAR aligner.

Immunohistochemistry (IHC) validation

As described previously, under the terms of the ethical approval, sections could only be cut for IHC from blocks of deceased participants (from melanoma or other causes) in order to avoid destruction of tumor blocks which might be required by the patient for testing in the future. A limited number of samples was therefore available. The staining and scoring were conducted as previously described [4]. Briefly, 5µm sections were mounted on Superfrost Plus slides (Thermo Fisher Scientific, UK), and underwent heat antigen retrieval, blocking, and

Hematoxylin counter-staining using IntelliPath FLX detection reagents (MenaPath, A. Menarini Diagnostics, UK). Primary antibodies were supplied by Abcam (UK): anti-MYC (ab32072), anti-NF-κB p105 (ab32360), anti-HLA-B (ab193415). Light microscopy (10X, 20X and 40X magnification) was used to evaluate protein expression. Staining scores were assigned to areas of the tissue section adjacent to the sampled region of the tumor (determined by the presence of a 'punch hole' in the tissue section). Tissue staining scores were recorded for cellular localization (nuclear, cytoplasmic and membranous) and intensity (0, no staining; 1, weak staining; 2, intermediate staining; 3, high intensity). In those tumors cored twice for RNA extraction (and hence had two 'punch holes'), 2 staining scores were generated and if these were conflicting, the slides were not used for subsequent analyses. Because the nuclear activity of NF-κB signaling is transient and not all the nuclei are positive at the same time, we undertook scoring as follows: if more than 5 nuclei were positive in the tumor we called the result "yes", otherwise a "no". For tumor infiltrating lymphocytes, cytoplasmic and nuclear staining we used a dichotomous "yes" or "no".

For the statistical analyses, the scores varying from 0 to 3 were pooled as follows: negative (0) and positive (1,2 or 3) in order to reduce random variation as the number of tumors scored positive was small for all targets measured. IHC scoring was conducted blinded and by pairs of independent observers (J. Pozniak and 1 other).

We used the light microscope and the Nuance software (v.3.0.1.2) to detect signals from haematoxylin, chromagen and the co-localised signal (both from haematoxylin and chromagen). The signals were recorded as the percentage of positive pixels per scoring area. The scoring areas were consistent for all of the samples. The scoring was performed using 20x magnification for the most representative part of the tumour (equivalent for HLA-B and MYC). The chromagen percentage only was recorded for HLA-B and the co-localised (nuclear) percentage signal for MYC.

CNA Analysis

Copy number profiles were estimated from next generation sequencing output from the DNA samples from the Leeds Melanoma Cohort primary tumour cores. The control (normal) germline DNA sequence data used were data generated from participants (n=312) from the Phase 3 of 1000 Genomes Project (ftp://ftp.1000genomes.ebi.ac.uk/vol1/ftp/phase3/data/) that matches the experimental set up (Illumina platform, low coverage, paired end library layout) were obtained. The bamwindow (https://github.com/alastair-droop/bamwindow) was used to create bins or windows of size 10k across the genome. Blacklisted regions (those for which sequence data were unreliable) were identified and excluded. The QDNASeq [5] package in R was used to identify highly variable regions in the genome which were added to

the blacklist and to adjust the read counts from each valid window based on the interaction of GC content and Mappability. The windows (10k) corresponding to a gene locus were identified using the R packages biomaRt and mcnv [6],[7]. The median value of the windows (10k) was used as the copy number estimate of a given gene. Because MYC covered only a one 10K window in the genome we chose to analyse a region commonly amplified in cancer around MYC [8]. We defined deletion and amplification as point cut-off of windows (covering a gene region) median at <=-0.3 or >=0.3 for the majority of the genes and at <=-0.45 or >=0.45 specifically for one gene (JAK2). Cut offs were decided based on testing the association of CNA and gene expression per given gene.

Power calculation

Our study was well-powered for all the analyses: for example, at α threshold 0.05, power was > 81% to detect differences in survival with hazard ratio>1.5 between identified tumour subgroups, > 80% to detect 1.4 fold change in mRNA expression between positive and negative stained slides or 1.2 fold change in mRNA expression by amplification or deletion and >88% to detect the effect of smoking similar to that observed.

References:

[1] Newton-Bishop JA, Beswick S, Randerson-Moor J, Chang YM, Affleck P, Elliott F, et al. Serum 25-hydroxyvitamin D3 levels are associated with Breslow thickness at presentation and survival from melanoma. J Clin Oncol 2009;27:5439–44. doi:10.1200/JCO.2009.22.1135.

[2] Newton-Bishop JA, Davies JR, Latheef F, Randerson-Moor J, Chan M, Gascoyne J, et al. 25-Hydroxyvitamin D2 /D3 levels and factors associated with systemic inflammation and melanoma survival in the Leeds Melanoma Cohort. Int J Cancer 2015;136:2890–9. doi:10.1002/ijc.29334.

[3] Raaijmakers MIG, Widmer DS, Maudrich M, Koch T, Langer A, Flace A, et al. A new live-cell biobank workflow efficiently recovers heterogeneous melanoma cells from native biopsies. Exp Dermatol 2015;24:377–80. doi:10.1111/exd.12683.

[4] Nsengimana J, Laye J, Filia A, O'Shea S, Muralidhar S, Poźniak J, et al. β-Catenin–mediated immune evasion pathway frequently operates in primary cutaneous melanomas. J Clin Invest 2018;128:2048–63. doi:10.1172/JCI95351.

[5] Scheinin I, Olshen AB, Reijneveld JC, Pinkel D, Albertson DG. DNA copy number analysis of fresh and formalin-fixed specimens by shallow whole-genome sequencing with identification and exclusion of problematic regions in the genome assembly n.d.:1–21.

[6] Durinck S, Spellman PT, Birney E, Huber W. Mapping identifiers for the integration of

genomic datasets with the R/ Bioconductor package biomaRt. Nat Protoc 2009;4:1184–91. doi:10.1038/nprot.2009.97.

[7] Durinck S, Moreau Y, Kasprzyk A, Davis S, De Moor B, Brazma A, et al. BioMart and Bioconductor: A powerful link between biological databases and microarray data analysis. Bioinformatics 2005;21:3439–40. doi:10.1093/bioinformatics/bti525.

[8] Zack TI, Schumacher SE, Carter SL, Cherniack AD, Saksena G, Tabak B, et al. Pan-cancer patterns of somatic copy number alteration. Nat Genet 2013;45:1134–40. doi:10.1038/ng.2760.