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Abstract

Synonymous mutations, which do not alter the protein sequence, have been shown to affect protein function (1-5). However, synonymous mutations are rarely investigated in the cancer genomics field. We used whole-genome and whole-exome sequencing to identify somatic mutations in 29 melanoma samples. Validation of one synonymous somatic mutation in *BCL2L12* in 285 samples identified 12 cases that harbored the recurrent F17F mutation. This led to increased *BCL2L12* mRNA and protein levels, due to differential targeting of wild-type and mutant *BCL2L12* by hsa-miR-671-5p. Protein made from mutant *BCL2L12* transcript bound p53, inhibited UV-induced apoptosis more efficiently than wild-type BCL2L12 and reduced endogenous p53 target gene transcription. This is the first report of positive selection of a recurrent somatic synonymous mutation in cancer. Our data indicate that "silent" alterations have a role to play in human cancer, emphasizing the importance of their investigation in future cancer genome studies.

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Systematic melanoma whole exome and whole genome studies have uncovered numerous recurrent mutations as well as highly mutated genes that show functional consequences on melanoma growth(6-11). These studies focus exclusively on coding mutations and specifically non-synonymous, insertion/deletion mutations, as well as splice sites. Recently non-coding mutations in the TERT promoter have been shown to generate new ETS binding motifs leading to increased activity of the TERT promoter (12, 13). These two recent studies highlight the importance of adjusting our focus beyond the non-synonymous coding mutations and evaluating all mutations in melanoma.

To gain further insight into the molecular alterations of melanoma, we report the analysis of 29 melanoma samples derived from treatment naïve patients and corresponding normal DNA. We performed whole-genome sequencing on ten matched normal and metastatic tumor DNAs and re-analyzed a previously published melanoma whole-genome study(14, 15). Together with our previous whole-exome analysis of 14 melanoma samples (6), and an additional whole-exome analysis of four matched melanoma and normal samples, this study allows for an unbiased search for novel melanoma genes in a total of 29 samples from treatment naïve patients.

Results

In combined analysis, a total of 13,098 somatic mutations were identified in genes. Of these, 8,619 caused protein changes, including 7,974 missense, 514 nonsense, 27 small deletions, 11 insertions and 93 at splice sites. There were 4,479 silent (synonymous) substitutions (Table S1). A non-synonymous to synonymous ratio (N/S) of 1.93:1, was calculated, which is not higher than the N/S ratio of 2.5:1 predicted for non-selected mutations (16), suggesting most are likely 'passenger' mutations. The number of C>T/G>A transitions was significantly greater than other nucleotide substitutions ($P < 0.001$) (Figure S1) consistent with an ultraviolet radiation (UVR) signature (17).

Recurrent non-synonymous mutations including BRAF V600E and TRRAP S722F substitutions were found (6, 18) as well as 16 recurrent synonymous mutations (Table 1). Although synonymous mutations do not alter the protein sequence, they have been shown to affect protein levels and function (2, 19). However, to date, synonymous mutations have not been investigated in numerous published cancer genomes. We sought to determine whether these somatic synonymous mutations may have a functional role in melanomagenesis. Further screening of these 16 synonymous hotspot mutations in an additional 169 melanoma samples identified *OR4C3* and *BCL2L12* (Figure S2) each to have identical synonymous mutations in three and four additional cases, respectively. The frequency of these recurrent alterations in the validation sample is significantly elevated ($p < 1x10^{-7}$ and $p< 1x10^{-11}$) suggesting they have either undergone relaxation of purifying selection (20) or have been under selection during

tumor development. As *BCL2L12* has previously been linked to tumorigenesis (21), we screened the *BCL2L12* cytosine to thymine change at position 51 (F17F) in another 87 melanoma samples. This identified six additional samples with the same alteration. This mutation thus occurred in 10/256 melanomas ($p < 1x10^{-31}$) in the combined validation study strongly suggesting it has a functional role in melanomagenesis. Consistent with this expectation, this nucleotide position displays evidence of selectionFigure S3), suggesting other nucleotide variants are not well-tolerated.

Synonymous mutations have been shown to affect gene function by multiple mechanisms including, but not limited to those exerting effects on mRNA splicing, protein translation and expression (5). Our analyses suggest that the synonymous alteration in *BCL2L12* does not affect splicing, as the mutation does not create a GT splicing consensus dinucleotide that could compete with the donor splice site (SS) of the first exon or encourage the use of seven cryptic GT splice donor sites within its vicinity (Table S2). Next, we determined whether *BCL2L12* allelic expression is affected by the mutation by comparing the levels of mutant and wild type *BCL2L12* alleles. We used MALDI-TOF (Sequenom) analysis to quantitatively assess relative allelic abundance in paired cDNA and gDNA from melanoma samples and found that for 9/12 samples the mutant BCL2L12 T allele was more abundantly expressed than the wild-type C allele (p- <0.01, Wilcoxon rank sum test)(Figure 1A-B). To test if the protein made from mutant *BCL2L12* transcript is expressed more abundantly than wild-type *BCL2L12*, we constructed wild-type and mutated versions of *BCL2L12* cDNA and transiently transfected them. We found that the mRNA (Figure S4) and protein levels (Figure 1C) of mutant *BCL2L12* were significantly increased relative to wild-type in multiple independent co-transfection experiments using GFP to control for transfection efficiency.

There could be a number of reasons leading to the elevated BCL2L12 protein expression levels: i) increased mRNA levels (noticed above), ii) enhanced mRNA translation, iii) stabilization of protein against degradation, or all of the above. We, however, found no change in elongation/translation rates of the mutant *BCL2L12* mRNA in comparison with the wild-type message (Figure S5) and no change in stability of protein (expressed *in vivo* and *in vitro*) towards limited proteolysis. These results largely rule out the F17F mutation having effects on BCL2L12 translation and protein stability.

The elevated levels of mutant *BCL2L12* mRNA could be due to increased transcription or increased RNA stability. The position corresponding to the mutation in *BCL2L12*, as well as a few neighboring synonymous sites, display high conservation across the mammalian lineage, suggesting functional constraints other than purely amino acid encoding (Figure S6A). However, position weight matrix (PWM) scanning coupled with chromatin immunoprecipitation (ChIP) analyses provide no support for a mechanism involving preferential binding of expressed transcription factors to the wild-type or mutated *BCL2L12* alleles (Figure S6B-E). Increased stability of the mutant *BCL2L12* mRNA could be due to differential binding of protein or miRNA to mutant and wild-type *BCL2L12* mRNA. Computational analysis showed that several RNA binding proteins may interact with wild-type and mutant mRNAs in the region close to the site of

mutation. However, gel-shift experiments of top candidate proteins did not reveal any differential binding between the two mRNAs (Figure S7).

Finally, to evaluate if the mutation affects miRNA binding we used the miRNA target prediction programs PITA (22) and miRanda (23). A single miRNA common to both programs, hsa-miR-671-5p, was predicted to bind the wild-type but not mutant *BCL2L12* transcripts. The miRNA target site in its wild-type form has high complementarity to mature hsa-miR-671-5p. Furthermore, Genome Evolutionary Rate Profiling (GERP) analysis, which identifies evolutionarily constrained positions in multiple genome alignments by quantifying substitution deficits across species, indicates that the target region exhibits high sequence conservation (Figure 2A). We hypothesized that loss of this target site in mutant *BCL2L12* may lead to increased *BCL2L12* transcript levels. hsa-miR-671-5p has previously been shown to be expressed in melanoma (24). Prior to targeting endogenous *BCL2L12* with hsa-miR-671-5p we used qRT-PCR analysis to detect the presence of transiently transfected miR mimic in melanoma cell lines (Figure 2B). Furthermore, to demonstrate specificity of the miR mimic to target wild-type *BCL2L12*, we co-transfected wild-type or mutant *BCL2L12* melanoma cell lines with negative control miR or hsa-miR-671-5p mimic in the presence of a specific miR inhibitor (anti-hsa-miR-671-5p). qRT-PCR analysis shows that anti-miR inhibited and reversed the effect on wild-type *BCL2L12* message by hsa-miR-671-5p. In mutant cell lines little to no effect was observed (Figure 2Ci-ii). The suppression of mature hsa-miR-671-5p mimic by co-transfection with anti-hsa-miR-671-5p was determined by qRT-PCR analysis (Figure S8). Our results indicate that wild-type *BCL2L12* mRNA is a target for hsa-miR-671-5p regulation which leads to its steady state reduction. However, the recurrent *BCL2L12* mutation reduces the affinity of hsa-miR-671-5p binding, thus allowing for mutant *BCL2L12* mRNA and protein accumulation.

BCL2L12 was previously shown to be amplified in glioblastoma, to bind p53 and to inhibit apoptosis (21). Together with our identification of a *BCL2L12* hotspot mutation that increases *BCL2L12* expression levels, this suggests BCL2L12 to be a candidate novel melanoma oncoprotein. We therefore investigated whether the identified *BCL2L12* C51T mutation may affect apoptosis.

As a first step in assessing this possibility we confirmed that the mutation does not interfere with p53 binding. Efficient complex formation between endogenous p53 and over-expressed protein transcribed from either wild-type or mutant *BCL2L12* transcript was seen in HEK293 cells (Figure S9). The observed interaction with p53 together with the enhanced expression of protein made from mutant *BCL2L12* transcript may repress p53 activity. This may lead to an increased ability of melanoma cells to resist p53-dependent induced apoptosis. To directly test this, we assessed the BCL2L12 anti-apoptotic activity after genotoxic stress in melanoma cells that either harbor wild-type or mutant *BCL2L12*. We used shRNA or siRNA to knock-down *BCL2L12* expression in *BCL2L12* wild-type (12T and SK-Mel-28) or mutant (75T, 79T, (specifically siRNA for 55T, and C025)) cells (Figures S10 and S11). In each case, the knockdown had little to no effect on cells harboring wild-type *BCL2L12* but significantly reduced the viability of cells harboring mutant *BCL2L12* post UVR exposure (Figure 3A-B). As BCL2L12 has

previously been shown to affect the expression of p53-dependent target genes (21), we tested if depletion of mutant *BCL2L12* in melanoma cells results in differential activation of p53-dependent transcription upon UVR treatment compared to *BCL2L12* depletion in wild-type expressing cells. Indeed, we observed significant increases in p53-dependent transcription of *MDM2* in mutant cells stably depleted of *BCL2L12* compared to stably depleted wild-type cells after UVR exposure (Figure 3C). Our results demonstrate that synonymous somatic mutations have important roles to play in cancer and, further suggest the potential for the pro-survival nature of BCL2L12 in melanoma.

Discussion

We identified a recurrent synonymous somatic mutation in *BCL2L12*. This mutation appears in 12 of 285 samples suggesting that this mutation is being selected for during tumor development. Analysis of the publicly available TCGA melanoma data set supports this as this alternative data set also contains this mutation. In the TCGA data this BCL2L12 mutation is found in 8 of 255 samples. Our study shows that the BCL2L12 synonymous mutation has no effect on normal protein function but instead causes an accumulation of *BCL2L12* mRNA and protein.

Functional analysis of the mutated form of BCL2L12 suggests the mutant's stability leads to over-expression increasing the anti-apoptotic signaling in melanoma cells and promoting cell survival, which may lead to increased resistance to p53-dependent apoptosis. BCL2L12 knockdown experiments support this finding. The reduced viability of mutant lines following BCL2L12 knockdown and UVR exposure suggest that these lines are dependent upon BCL2L12 expression for survival, a common occurrence known as oncogene addiction(25) . In fact for several of the mutant lines stable knockdown of BCL2L12 lead to cell death without any exposure to UVR further supporting the role of BCL2L12 in tumor survival.

A similar role between altered miRNA binding and synonymous mutations has been shown previously. Where a synonymous mutation in the immunity-related-GTPase-family-M (IRGM) gene altered miR-196 binding and deregulated IRGM-dependent-xenophagy in Crohn's disease, implicating a synonymous mutation as a likely causal variant for this disease(26). However, a direct link between a synonymous mutation and the origin of cancer has not been shown. This study thus demonstrates for the first time that synonymous mutations may be selected in cancer and play a role in tumorigenesis. Importantly, the selection mechanism may possibly be via the relaxation of purifying selection and/or plasticity-relaxation-mutation (PRM) mechanism as well as some other alternatives (20, 27-31) rather than positive selection. The data presented here cannot unambiguously select one specific mechanism. However, the presented genetic and functional data supports our view that synonymous mutations should receive increasing attention, not only in their detection, but also in their functional assessment and elucidation of their role in cancer.

Accession codes. *BRAF*, CCDS5863.1; *TRRAP*, CCDS5659.1; *OR4C3* CCDS31489.1; *BCL2L12*, CCDS12776.1; p53, CCDS11118.1; MDM2, CCDS8986.2.

Disclaimer

The findings and conclusions in this article have not been formally disseminated by the Food and Drug Administration and should not be construed to represent any Agency determination or policy.

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Author contributions

S.C.J. P., J.J.G., E.H.M., T.D.P., C.K.S., A.A.K., and Y.S. designed the study; K.S.H., M.A.D., J.E.G., W.R., S.R., U.K.B., K.D., N.K.H. and S.A.R. collected and analyzed the melanoma samples; S.C.J.P., J.J.G., X.W., J.K.T., J.C.L., K.D., N.K.H., V.G., H.C., R. K., N.K., and the NISC Comparative Sequencing Program, analyzed the genetic data. T.D.P, V.S., S.J., performed the functional analyses. M.L.S, M.A.M, and F.S.C, designed performed and analyzed Sequenom expression and ChIP assay. All authors contributed to the final version of the paper.

Data Access

Somatic variants are listed in Table S1, and will be deposited to dbSNP. http://www.ncbi.nlm.nih.gov/projects/SNP/

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Figure Legends

Figure 1. Abundance of the *BCL2L12* transcript and BCL2L12 protein. A. Image of MALDI-TOF (Sequenom) spectrographs indicating peaks for unextended primer (arrow), C allele (filled arrowhead), and T allele (open arrowhead) for sample 55T cDNA. Unextended primer peak in the –RT control confirms that allelic representation differences in cDNA samples are not due to genomic DNA (gDNA) contamination. Paired genomic DNA from each sample was used as a control. **B.** Box plots demonstrate significantly higher T allele representation in cDNA (blue) compared to gDNA (gray) in 9/12 melanoma samples. Significance was calculated from 12 measurements of each cDNA and gDNA sample using the Wilcoxon rank sum test; asterisks indicate samples with $p<0.01$. **C**. BCL2L12 protein levels in transiently transfected HEK293T cells (western blot analysis). Cells were transiently transfected with *BCL2L12* wild-type or mutant cDNAs and western blotting was done post-transfection.

Figure 2. hsa-miR-671-5p represses wild-type *BCL2L12* expression. A. Schematic representation of the *BCL2L12* locus at hg18 coordinates chr19:54860211-54868985. Based on miRanda and PITA target scanning predictions, hsa-miR-671-5p binds at the first coding exon and has high affinity to the wild-type version but not the C51T version. GERP single-nucleotide resolution evolutionary conservation scores show that this region is highly conserved. The horizontal line at GERP score = 2 indicates the general threshold that defines evolutionarily constrained bases. For this plot we only show GERP scores >= 0. **B.** qRT-PCR analysis of precursor hsa-miR-671 in melanoma cells. Graphs show experimental replicates of qRT-PCR analysis of precursor hsa-miR-671 in mutant *BCL2L12* (75T and 79T) compared to wild-type *BCL2L12* (12T and SK-Mel-28) cell. Results are representative of two independent experiments. Error bars, sd. **C.** Anti-miR-671-5p rescues hsa-miR-671-5p mediated knock-down of wild-type BCL2L12 in melanoma cells. Graphs show experimental replicates of qRT-PCR of endogenous BCL2L12 levels in mutant *BCL2L12* (75T and 79T) and wild-type *BCL2L12* (12T and SK-Mel-28) cell lines in the presence of negative control miR (NC) or hsa-miR-671-5p (miR) plus 0nM or 50nM anti-miR-671-5p. Results are representative of two

independent experiments. Error bars, sd. Comparison of NC0 to miR0 * $p<0.01$ or comparison of miR0 to miR50 ** $p<0.04$; students t-test.

Figure 3. Effects of the *BCL2L12* (C51T; F17F) recurrent mutation on BCL2L12 function. **A.** Graphical representation showing the anti-apoptotic effect of BCL2L12 mutant cells compared to wild-type BCL2L12 cells post UV treatment. The relative cell numbers after the cells were treated for 48 hr with UV as estimated by CellTiter-Glo and plotted as percent survival, Error bars, sd. **B.** Melanoma cells ((wt) 12T, SK-Mel-28; (mut) 75T, 79T, 55T, or C025) transiently transfected with *BCL2L12* specific siRNA were tested for sensitivity to UV induced cell death. Shown are respresentative graphs from all cell lines exposed to 50KμJ of UV light. Results were analyzed using Microsoft Excel and GraphPad Prism v5 and graphs are representative of experimental replicates.. Error bars, sd (*$p<0.04$ comparing siRNA to NC).**C.** qRT-PCR analysis shows that depletion of mutant forms of *BCL2L12* using specific shRNA increases p53-dependent target gene expression compared to depletion of wild-type *BCL2L12*. Graphs show qRT-PCR analysis of wild-type *BCL2L12* (12T) and mutant *BCL2L12* (75T) pooled clone mRNA expression levels for *Mdm2*. Results shown are experimental replicates analyzed using student's unpaired t-test. Error bars, sd.