CELL CYCLE
2016, VOL. 15, NO. 16, 2157–2163
http://dx.doi.org/10.1080/15384101.2016.1195532

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REPORT

TCGA: Increased oncoprotein coding region mutations correlate with a greater expression of apoptosis-effector genes and a positive outcome for stomach adenocarcinoma

John M. Yavorski[a] and George Blanck[a,b]

[a]Department of Molecular Medicine, Morsani College of Medicine, University of South Florida, Tampa, FL, USA; [b]Immunology Program, H. Lee Moffitt Cancer Center and Research Institute, Tampa, FL, USA

ABSTRACT

Oncogene mutations are primarily thought to facilitate uncontrolled cell growth. However, overexpression of oncoproteins likely leads to apoptosis in a feed forward mechanism, whereby a certain level of oncoprotein leads to the activation of pro-proliferation effector genes and higher levels lead to activation of pro-apoptotic effector genes. TCGA STAD barcodes having no oncoprotein coding region mutations represented reduced expression of the apoptosis-effector genes compared with barcodes with multiple oncoprotein coding region mutations. Furthermore, STAD barcodes in a "no-subsequent tumor" group, representing 224 samples, and in a "positive outcome" group, had more oncoprotein coding regions mutated, on average, than barcodes of the new tumor and negative outcome groups, respectively. BRAF, CTNNB1, KRAS and MTOR coding region mutations (as a group) had the strongest association with the no-subsequent tumor group. Tumor suppressor coding region mutations were also correlated with no-subsequent tumor. These results are consistent with an oncoprotein-mediated, feed-forward mechanism of apoptosis in patients. Importantly, the no-subsequent tumor group also had more overall mutations. This result leads to considerations of unhealthy cells or cells with more neo-antigens for immune rejection. However, a probabilistic aspect of mutagenesis is also consistent with more oncoprotein and tumor suppressor protein mutations, in cases of more overall mutations, and thus a higher likelihood of activation of feed forward apoptosis pathways.

ARTICLE HISTORY
Received 8 April 2016
Revised 18 May 2016
Accepted 22 May 2016

KEYWORDS
apoptosis-effector genes; feed-forward apoptosis; oncoprotein mediated apoptosis; stomach adenocarcinoma; tumor suppressor proteins

Introduction

The capacity of oncoproteins to stimulate pro-proliferative effector genes and cell division has overwhelmed cancer research. However, oncoproteins also have the capacity to effect apoptosis, particularly at high levels of expression. There are numerous settings where the pro-apoptotic role for what have traditionally been referred to as oncoproteins comes into effect. For example, E2F1 knock-out mice develop tumors,[1,2] despite the role of E2F1 in activating pro-proliferation effector genes such as the histone genes and dihydrofolate reductase.[3-5] Over-activation of the T-cell receptor, in the course of deletion of self-reactive T-cells, in the thymus, leads to T-cell apoptosis.[6-8] Over-expression of POU2F1 (Oct1) leads to apoptosis,[9-13] although POU2F1 activates histone genes.[14,15] Overexpression of NF-kappaB leads to blast cell apoptosis in anaplastic large cell lymphoma.[16,17]

While there are several potential mechanisms for the pro-apoptotic effect of overexpressed, pro-proliferative regulatory proteins,[18,19] a very credible possibility is represented by the difference in the gene sizes of pro-proliferative versus pro-apoptotic effector genes. McKay et al.[20] in particular noticed the unusually small size of apoptosis genes, and later work by our group confirmed the relative size relationships between pro-proliferative and apoptosis-effector genes.[21] Furthermore, it has been demonstrated that pro-proliferative effector genes are much bigger "sinks" for pro-proliferative transcription factors, e.g., MYC.[21] Pro-proliferative effector genes also have more regions of open chromatin, i.e., more functionally effective regulatory regions than do apoptosis-effector genes.[21,22]

The above data and paradigm are well represented in cancer medicine by only one case, whereby it has been established that MYCN amplification represents a relatively good prognosis for neuroblastoma specifically in cases where the caspase 8 apoptosis-effector gene is present.[23-27] This case prompted us to re-address this issue but by focusing on activating mutations rather than amplification, particularly after having noted that there are patient cases where there are multiple oncoprotein mutations and other cases where cancer develops in the absence of any known oncoprotein mutation, a relatively unexpected situation considering the comprehensive wealth of knowledge known about the available oncoproteins and their considerable role in the theory of oncogenesis. The results for the analysis of the TCGA STAD set indicate that indeed, more oncoprotein mutations correlate with a more favorable outcome.

CONTACT George Blanck gblanck@health.usf.edu 12901 Bruce B. Downs Bd. MDC7, Tampa, FL 33612, USA.
Supplemental data for this article can be accessed on the to publisher's website.

Table 1. HUGO symbols for the oncoprotein, tumor suppressor and apoptosis-effector protein coding region sets used in the analyses. (See SOM file labeled, "SOM Table 1, source file.").

Oncoproteins	Tumor suppressors		Apoptosis-effector genes	
ACVR1	AKAP12	KISS1R	AIFM2	COX6A1
ALK	AXIN1	KLF6	BAD	COX6B1
ARAF	BMP2	LATS2	BAX	COX7A2L
BRAF	BMPR1B	LIMD1	BRCA1	COX7C
CTNNB1	BMPR2	MAP2K4	CASP12	CRADD
EGFR	BRCA1	MED23	CASP3	CYC1
FGFR2	BRCA2	PBRM1	CASP4	GZMA
FLT3	BRMS1	PEBP1	CASP5	GZMB
FRK	CASZ1	PPAPDC1B	CASP7	LOC643733
HRAS	CDKN2A	PRDM2	CASP9	PARP1
JAK2	CHD5	PTEN	CHEK1	SIVA1
KRAS	CHEK2	RB1	COX5A	UQCRC2
MTOR	CTCF	RECK	COX5B	
NRAS	DLC1	SMAD4		
PRKACA	DOK2	SMAD7		
RAF1	FLCN	SMARCB1		
	FOXP3	SP100		
	GPR68	TFPI2		
	ING1	TMPRSS11A		
	ING4	TXNIP		
	INPP4B	VHL		
	KISS1	WWOX		

Results

Apoptosis-effector gene expression for the TCGA STAD dataset was compared for barcodes having mutations in 4 or more oncoprotein coding regions vs. 3 independent sets of randomly selected barcodes having no oncogene, coding region mutations (Table 1). For the subsequent analyses in this report, we grouped the oncoprotein and tumor suppressor coding regions, keeping in mind the clear degeneracy of aberrant signaling pathway activation that sustains cancer growth, particular apparent recently with the activation of alternative signaling pathways in designer drug resistant cancer cells.[28,29] The barcodes with oncoprotein coding region mutations had a greater level of RNA expression for a previously and rigorously defined set of apoptosis-effector genes[21,22,30] compared to the randomly selected barcodes lacking mutations in the indicated oncogene set (Tables 1, 2; Random Set 1 $p < 0.018$, Set 2 $p < 0.018$, Set 3 $p < 7.76E-05$).

To determine whether there was a clinical distinction between more or fewer oncoprotein coding region mutations, we established groups of barcodes from the STAD and BLCA cancer sets, as stated above, according to the clinical outcome methods 1 and 2 (see Methods): the development of a new tumor versus no-subsequent tumor; and negative and positive (treatment) outcome, respectively. The groups were compared based on the number of oncoprotein coding regions mutated, i.e. the coding region for that barcode was mutated at least once (Fig. 1A, B). The no-subsequent tumor and positive outcome groups for the STAD data set had a higher number of oncoprotein coding regions mutated ($p < 0.004$ and $p < 0.026$, respectively), while the BLCA cancer set did not show any differences in oncoprotein coding region mutations for the 2 different clinical outcome groups.

Inspection of the new tumor and no-subsequent tumor data for the STAD dataset revealed 4 oncoprotein coding regions (BRAF, CTNNB1, KRAS and MTOR) that were mutated in the greatest number of barcodes (Table 3). A difference in the number of mutations representing this group of oncoproteins, between the new tumor and no-subsequent tumor barcodes, represented a statistical significance of $p < 2.24E-05$.

Mutation rates for the oncoprotein coding region set were recalculated to consider all mutations in a given coding region (rather than whether the coding region was "hit" or not), along with the mutation rates for the tumor suppressor gene set and total genome mutations. A comparison of the new tumor and no-subsequent tumor barcodes showed an increase in the average number of oncoprotein, tumor suppressor and total coding region mutations ($p < 0.007$, $p < 0.019$ and $p < 0.004$, respectively) for the no-subsequent tumor group in the STAD data set (Fig. 2A–C). The BLCA cancer sets representing new tumor

Table 2. Comparison of the expression of apoptosis-effector genes in the samples representing the high oncoprotein mutation barcodes vs. randomly selected barcodes having no oncoprotein mutations. (See SOM file labeled, "SOM STAD, apoptosis.").

	Barcodes w/ high oncoprotein mutations	Barcodes w/ no oncoprotein mutations SET 1	Barcodes w/ no oncoprotein mutations SET 2	Barcodes w/ no oncoprotein mutations SET 3
	TCGA-BR-8680	TCGA-HU-A4G3	TCGA-BR-A4IU	TCGA-D7-8570
	TCGA-BR-6452	TCGA-D7-6818	TCGA-BR-6801	TCGA-IN-8462
	TCGA-VQ-A8P2	TCGA-CD-5800	TCGA-BR-A4J7	TCGA-FP-A9TM
	TCGA-CG-5721	TCGA-IN-A6RS	TCGA-CD-5798	TCGA-MX-A663
	TCGA-BR-8487	TCGA-HU-A4GP	TCGA-BR-8590	TCGA-BR-8373
	TCGA-FP-A4BE	TCGA-BR-8291	TCGA-RD-A8MW	TCGA-VQ-AA6B
	TCGA-CD-A4MG	TCGA-VQ-A92D	TCGA-CD-8530	TCGA-BR-8683
	TCGA-CG-5723	TCGA-HU-A4H6	TCGA-D7-8574	TCGA-BR-A4CS
	TCGA-HU-8602	TCGA-CD-8534	TCGA-CD-5799	TCGA-D7-8579
	TCGA-BR-8361	TCGA-BR-8384	TCGA-BR-8367	TCGA-BR-A4IV
	TCGA-BR-8363	TCGA-BR-8364	TCGA-BR-8289	TCGA-D7-A6F0
	TCGA-BR-8589	TCGA-CG-5724	TCGA-ZA-A8F6	TCGA-RD-A8N9
	TCGA-D7-A4YX	TCGA-HU-8604	TCGA-BR-8588	TCGA-BR-A4CR
Average apoptosis gene expression (Avg. RPKM)	100.09	70.57	70.93	54.91
P-value comparison of SET # vs. high oncoprotein mutation group	N/A	0.018	0.018	7.76E-05

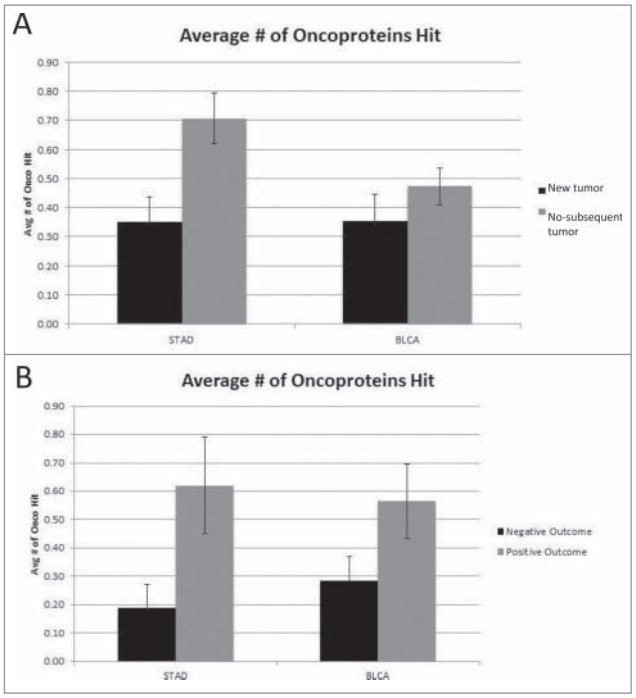

Figure 1. (A) Average number of oncoproteins mutated for New Tumor vs. No-subsequent tumor (STAD $p < 0.004$, BLCA not significant) (detailed in SOM file labeled, "SOM Figures 1, 2 and 3, source file"). The new tumor group represents 74 barcodes and the no-subsequent tumor group represents 224 barcodes. (B) Average number of oncoproteins mutated for Negative vs. Positive outcome (STAD $p < 0.026$, BLCA not significant) (detailed in SOM file labeled, "SOM Figures 1, 2 and 3, source file"). The negative outcome group represents 32 barcodes and the positive outcome group represents 71 barcodes.

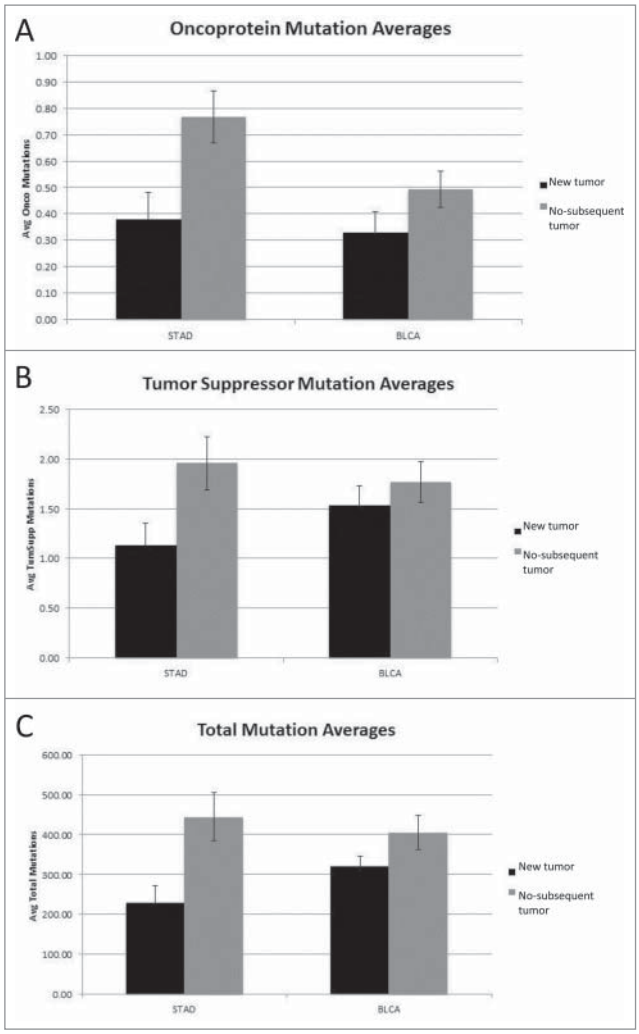

Figure 2. (A) Oncoprotein mutation averages for New Tumor vs. No-subsequent tumor (STAD $p < 0.007$, BLCA not significant) (detailed in SOM file labeled, "SOM Figures 1, 2 and 3, source file"). The new tumor group represents 74 barcodes and the no-subsequent tumor group represents 224 barcodes. (B) Tumor suppressor mutation averages for New Tumor vs. No-subsequent tumor (STAD $p < 0.019$, BLCA not significant) (detailed in SOM file labeled, "SOM Figures 1, 2 and 3, source file"). The new tumor group represents 74 barcodes and the no-subsequent tumor group represents 224 barcodes. (C) Total mutation averages for New Tumor vs. No-subsequent tumor (STAD $p< 0.004$, BLCA not significant) (detailed in the SOM file labeled, "SOM Figures 1, 2 and 3, source file"). The new tumor group represents 74 barcodes and the no-subsequent tumor group represents 224 barcodes.

and no-subsequent tumor, respectively, did not differ significantly in the number of oncoprotein, tumor suppressor or total genome mutations. Using the same approach of considering all mutations, a comparison of the negative and positive outcome barcodes was conducted (Fig. 3A–C). The positive outcome group in the STAD dataset had a higher average number of oncoprotein coding region mutations compared to the negative outcome group ($p < 0.042$).

Previous work has indicated that the ratio of silent to amino acid altering mutations is highly constant within a TCGA cancer data set, e.g., regardless of whether a barcode has few mutations or a large number of mutations.[31] That result is consistent with the overwhelming stochastic nature of somatic mutagenesis. Thus, to a very significant degree, total mutations track amino acid altering mutations. Regardless, we repeated the above analyses with all of the silent mutations removed (Table 4). The new tumor vs. no-subsequent tumor, and negative versus positive outcome groups, were compared for the oncoprotein, tumor suppressor protein and total coding region mutation sets. As expected, the above described, statistically significant correlations of STAD oncoprotein, tumor suppressor protein, and overall coding region mutations, with the indicated clinical parameters, were reproduced and verified.

To further consider the functional impact of the higher level of mutations correlating with a favorable course of disease, the number of deleterious amino acid changes for the barcodes representing the new tumor vs. no-subsequent tumor, and negative versus positive outcome, were determined for the tumor suppressor coding region set in the STAD cancer data set (Table 5). The no-subsequent tumor and positive outcome groups show

Table 3. Reduced oncoprotein set for comparison of the new tumor vs. no-subsequent tumor in the STAD data set. Comparison made based on the oncoprotein being mutated at least once or not (detailed in the SOM file labeled, "SOM STAD, new tumor_onco hit or not"). The p-value represents more oncoprotein mutations in the no-subsequent tumor group. The new tumor group represents 74 barcodes and the no-subsequent tumor group represents 224 barcodes.

	Reduced Oncoprotein Set (Hit or Not)
	BRAF
	CTNNB1
	KRAS
	MTOR
P-value	2.24E-05

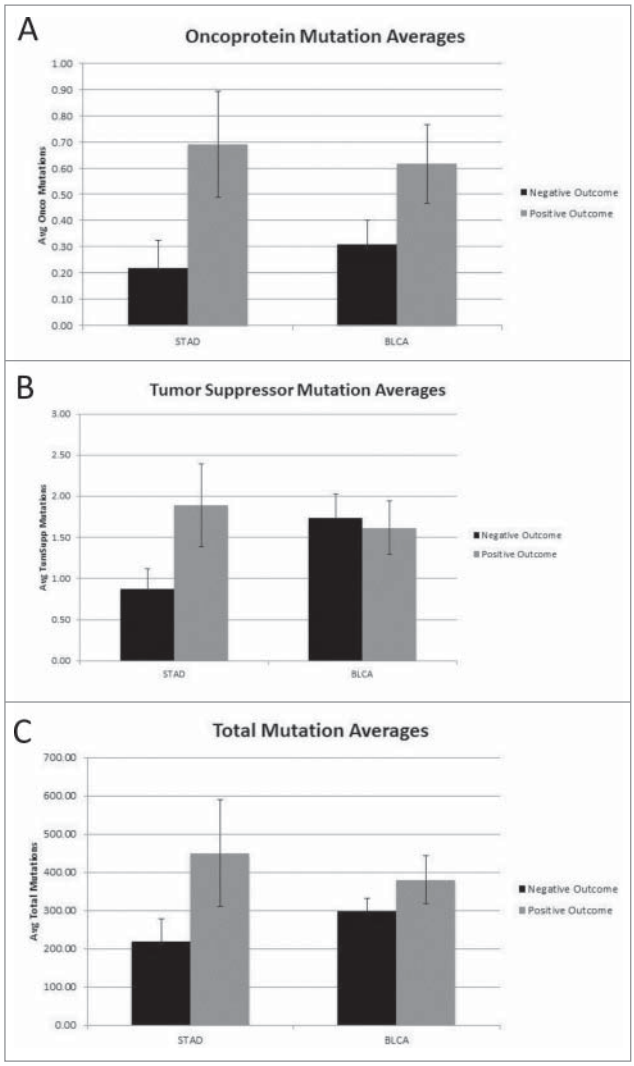

Figure 3. (A) Oncoprotein mutation averages for Negative and Positive outcome (STAD $p < 0.042$, BLCA not significant) (detailed in SOM file labeled, "SOM Figures 1, 2 and 3, source file"). The negative outcome group represents 32 barcodes and the positive outcome group represents 71 barcodes. (B) Tumor suppressor mutation averages for Negative and Positive outcome (Both STAD and BLCA are not significant) (detailed in SOM file labeled, "SOM Figures 1, 2 and 3, source file"). The negative outcome group represents 32 barcodes and the positive outcome group represents 71 barcodes. (C) Total mutation averages for Negative vs. Positive outcome (STAD and BLCA are not significant) (detailed in SOM file labeled, "SOM Figure 1, 2 and 3, source file"). The negative outcome group represents 32 barcodes and the positive outcome group represents 71 barcodes.

an approximate 2-fold increase in the number deleterious amino acid changes compared to the new tumor and negative outcome groups, respectively. The number of deleterious amino acid changes was also calculated for the oncoprotein gene set in the STAD cancer dataset. The no-subsequent tumor and positive outcome groups, again, showed an approximate 2-fold increase in the number of deleterious amino acid changes compared to the new tumor and positive outcome groups, respectively (Table 5).

Discussion

For decades, there has been an apparent contradiction with regard to the dual functions of oncoproteins and pro-proliferative signaling pathways in proliferation and apoptosis. There is very little acceptance in the scientific community regarding a mechanism to unify these functions, but one very credible proposal is that over-expression or over-activation of oncoproteins leads to apoptosis. In addition to the extensive data consistent with this mechanism, the mechanism represents a certain biologic elegance, in that apoptosis largely results from a failure to transit S-phase, and such a failure may prevent the negative feedback that would ordinarily be in effect with the completion of cell-division that would dampen pro-S-phase activation processes. Without the negative feedback, S-phase activation processes continue. The prolongation of oncoprotein-mediated, S-phase stimulation processes, e.g., prolonged because of the absence of needed metabolites and S-phase block, would be conveniently exploited to engineer cell apoptosis when practical for the organism, because of the pending S-phase failure.

Despite the extensive scientific literature confirming the feed-forward mechanism of apoptosis, regardless of the debate over molecular details, very little of this knowledge has led to progress in cancer patient treatments or prognosis, almost certainly because of the complexity of assessing and exploiting degrees of oncoprotein expression or activity, as opposed to the discreet, presence or absence of an oncoprotein. Interestingly, decades ago, high dose estrogen strategy was employed for breast cancer treatment,[32] possibly due to hints of breast cancer cell susceptibility with over-activation of what would otherwise be a pro-proliferative pathway. And, as indicated above, MYCN amplification has been noted as prognostic of a more favorable outcome for neuroblastoma. And, it is relatively common for rapidly dividing tumors to respond well to therapy.[33,34] Such results have been attributed to the assumption that rapidly dividing cells rapidly acquire a high mutation burden from mutagenic therapy. However, it remains possible that rapidly dividing cells are close to the tipping point, with regard to over-activation of oncproteins, thereby being well poised to enter apoptosis following mutagenic therapy that halts or

Table 4. Indications of the p-values for comparison of mutation occurrences, in the oncoprotein, tumor suppressor protein and total coding region sets, for the STAD dataset, when silent mutations are either included or removed. (These data are compiled from the SOM files labeled, "SOM STAD, new tumor_results" and "SOM STAD, neg and pos outcome_results.") In all cases, the p-values represent higher numbers of mutations in the no-subsequent tumor group and in the positive outcome group. The new tumor group represents 74 barcodes and the no-subsequent tumor group represents 224 barcodes. The negative outcome group represents 32 barcodes and the positive outcome group represents 71 barcodes.

	Oncoprotein		Tumor Suppressor		Total	
	New Tumor vs No-subsequent tumor	Neg vs Pos Outcome	New Tumor vs No-subsequent tumor	Neg vs Pos Outcome	New Tumor vs No-subsequent tumor	Neg vs Pos Outcome
Silent Included	0.007	0.041	0.018	0.072	0.004	0.129
Silent Removed	0.017	0.036	0.026	0.104	0.004	0.128

Table 5. Average number of deleterious amino acid changes per barcode for each clinical outcome group for the STAD cancer set using PROVEAN (data compiled from the SOM files labeled, "SOM STAD, PROVEAN_new tumor_onco," "SOM STAD, PROVEAN_new tumor_tumsupp," "SOM STAD, PROVEAN_negandpos outcome_onco," "SOM STAD, PROVEAN_neg and pos outcome_tumsupp"). The new tumor group represents 74 barcodes and the no-subsequent tumor group represents 224 barcodes. The negative outcome group represents 32 barcodes and the positive outcome group represents 71 barcodes.

	Average # of deleterious AA changes per barcode			
	New Tumor	No-subsequent tumor	Negative Outcome	Positive Outcome
Tumor Suppressor	0.35	0.63	0.31	0.73
Oncoprotein	0.20	0.38	0.09	0.34

significantly delays S-phase. Given the likely complications involved in treating patient tumors with drugs meant to stimulate tumor growth, in the hopes of "over-treatment" and driving tumor cells into apoptosis, a feed-forward mechanism of apoptosis does not immediately suggest therapeutic options. But it is interesting to note that decades ago, high dose estrogen therapies were used to treat breast cancer.[32]

For the first time, the above analysis indicates a likely setting whereby more oncoprotein coding region mutations represent a better outcome, in retrospect not surprising given the pioneering work indicating the association of MYCN amplification with a better outcome and the decades old work indicating that rapidly dividing cancers are more vulnerable to chemotherapy.

The oncoprotein coding region mutation associations with a more favorable disease course for STAD is verified by analogous results presented above for tumor suppressor protein coding region mutations. However, the results did not apply to the BLCA data set, either because of distinct cancer development mechanisms or simply for technical reasons, such as the use of an oncoprotein set that is not as relevant to BLCA as STAD.

Methods

Apoptosis-effector gene expression, oncoprotein set, tumor suppressor set

Clinical and somatic mutation data were collected for the stomach adenocarcinoma (STAD) dataset from the TCGA data portal (http://cancergenome.nih.gov/), under NIH/dbGAP project approval number #6300. Tumor sample barcodes in the somatic mutation (exome) file were truncated to contain only the following characters, TCGA-##-####. Mutation data from the comprehensive mutation file, including truncated tumor sample barcodes and mutation type (amino acid altering or silent), were collected for the oncoprotein and tumor suppressor coding region sets (HUGO symbols listed in Table 1) and a Microsoft Excel COUNTIF function was used to determine the number of oncoprotein coding region mutations per barcode for the barcodes collected from the clinical patient file. The oncoprotein and tumor suppressor sets have been previously described.[31,35,36] Barcodes having 4 or more oncoprotein coding region mutations were considered to be high oncoprotein coding region mutation barcodes and served as the experimental group. Barcodes having no known oncoprotein coding region mutations, based on the above referenced oncoprotein set, were sorted randomly using a random number generator function and grouped into 3 sets (SET 1-3) to serve as control groups ("SOM STAD, apoptosis").

Level 3 BCGSC IlluminaHiSeq RNASeq data was collected from the TCGA data portal for the barcodes in the experimental group and each of the 3 control groups ("SOM STAD, apoptosis"). From the gene quantification file, RPKM values of the apoptosis-effector gene set (Table 1) were collected for each barcode. This (Table 1) set of apoptosis genes is referred to as "Set A" in several previous publications,[21,22,30] and represents a high stringency standard for the definition of apoptosis-effector gene, as described.[21,22,30] However, due to issues related to data availability for the STAD RNASeq files, the following 3 genes were eliminated from the apoptosis-effector gene set described in refs. 21, 22, 30: COX6B2, COX7B2, and AIFM3.

Clinical outcome method 1: Developing new tumor vs. no-subsequent tumor

The TCGA STAD clinical follow up file was used to categorize barcodes based on the new tumor event data. Barcodes having developed a new tumor status post initial treatment were grouped and labeled "new tumor," and those not having developed a new tumor were grouped and labeled "no-subsequent tumor." Any barcode with no associated mutation data was eliminated and a comparison between the remaining new tumor and no-subsequent tumor barcodes was conducted based on the total number of oncoprotein coding regions mutated, i.e., based on the coding region being mutated at least once. (Additional detail of methods is provided in SOM file labeled, "SOM STAD, new tumor_onco hit or not"). We then considered the total number of mutations, i.e. included all mutations occurring in each coding region per barcode, and compared the new tumor and no-subsequent tumor barcodes. Using this same approach of considering all mutations per coding region, we compared the new tumor and no-subsequent tumor barcodes based on the tumor suppressor gene set (Table 1) and total mutation rates (Additional detail of method provided in SOM file labeled, "SOM STAD, new tumor results").

Clinical outcome method 2: Treatment best response

The TCGA STAD clinical drug file was used to categorize barcodes into negative and positive outcome groups based on the treatment best response data. Barcodes with stable or clinical progressive disease were grouped and labeled "negative outcome," while barcodes with partial or complete response were grouped and labeled "positive outcome." Any barcode appearing in multiple treatment, best response categories or with no corresponding mutation data was eliminated. The above methods of comparison outlined in clinical outcome method 1 were used to compare the negative and positive outcome groups. (Additional detail for methods is provided in SOM files labeled, "SOM STAD, neg and pos outcome_onco hit or not" and "SOM STAD, neg and pos outcome_results.") The above methods for clinical outcome methods 1 and 2 were then repeated for the TCGA BLCA cancer set from TCGA. The corresponding SOM

files are labeled, "SOM BLCA," with the same corresponding suffix as used for the STAD SOM files.

Deleterious amino acid changes: PROVEAN

The average number of deleterious amino acid changes was determined for each clinical outcome group, i.e., new tumor, no-subsequent tumor, negative outcome and positive outcome, for the oncoprotein and tumor suppressor coding region sets for the STAD data set. The chromosome number, start position, reference allele and tumor sequence allele data were collected for the oncoprotein and tumor suppressor coding region sets[31,35,36] for each clinical outcome group. The data was then copied into PROVEAN under the "Human Genome Variants" protocol. The removal of duplicates from the "#ROW_NO." column in the PROVEAN output was used to determine the number of deleterious amino acid changes for the oncoprotein and tumor suppressor coding region sets for each of the clinical outcome groups. The number of deleterious amino acid changes was then divided by the sample size to determine the average number of deleterious amino acid changes per barcode for each clinical outcome group for the STAD cancer dataset (Additional detail provided in the SOM files labeled, "SOM STAD, PROVEAN_new tumor_onco," "SOM STAD, PROVEAN_new tumor_tumsupp," "SOM STAD, PROVEAN_neg and pos outcome_onco," and "SOM STAD, PROVEAN_neg and pos outcome_tumsupp").

Disclosure of potential conflicts of interest

No potential conflicts of interest were disclosed.

Funding

Authors acknowledge the financial contributions of the Anna Valentine Fund and the taxpayers of the State of Florida.

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