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PERSPECTIVE

Haploinsufficiency of Tumor Suppressor Genes is Driven by the Cumulative Effect of microRNAs, microRNA Binding Site Polymorphisms and microRNA Polymorphisms: An In silico Approach

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Abstract: Haploinsufficiency of tumor suppressor genes, wherein the reduced production and activity of proteins results in the inability of the cell to maintain normal cellular function, is one among the various causes of cancer. However the precise molecular mechanisms underlying this condition remain unclear. Here we hypothesize that single nucleotide polymorphisms (SNPs) in the 3′untranslated region (UTR) of mRNAs and microRNA seed sequence (miR-SNPs) may cause haploinsufficiency at the level of proteins through altered binding specificity of microRNAs (miRNAs). Bioinformatics analysis of haploinsufficient genes for variations in their 3′UTR showed that the occurrence of SNPs result in the creation of new binding sites for miRNAs, thereby bringing the respective mRNA variant under the control of more miRNAs. In addition, 19 miR-SNPs were found to result in non-specific binding of microRNAs to tumor suppressors. Networking analysis suggests that the haploinsufficient tumor suppressor genes strongly interact with one another, and any subtle alterations in this network will contribute to tumorigenesis.

Keywords: haploinsufficiency, microRNA, single nucleotide polymorphism, miR-SNPs, tumor suppressor genes, cancer

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Introduction

Cancer is a complex genetic disease involving structural and expression abnormalities of both coding and non-coding genes. The search for cancer causing genes has identified two major classes namely the oncogenes and tumor suppressor genes (TSGs). Activation or over expression of a proto-oncogene, such as *cMyc*,[1] or inactivation of a tumor suppressor, such as *TP53*,[2,3] causes tumorigenicity. Oncogene activation in tumors is straightforward, while the inactivation of TSGs is a complex process attained by different means including mutations, deletions, down regulation by microRNAs, epigenetic silencing etc. Although complete loss of TSG is common in tumors, recent studies indicate that a partial compromise in TSG function termed 'haploinsufficiency' contributes to the development and progression of many cancers.[4] The condition where one functional allele of a gene is lost by mutation or deletion, and the remaining normal allele is insufficient to execute its original physiological function, is called haploinsufficiency. This phenomenon is extensively applicable to TSGs[5] as they offer a benefit for cancer cells in regards to proliferation,[6] survival,[7] and metastasis.[8]

In principle, haploinsufficient TSGs are impaired by a 50% reduction in expression or activity. However, *in vivo* studies demonstrate that even a subtle 20% reduction of *PTEN* protein level—termed as 'quasi-insufficiency'—could contribute to the development of cancer.[9] Another example is that *TP53*, when targeted by short hairpin RNAs (shRNAs), is shown to elicit distinct phenotypes ranging from hyperplasia to malignancy in mouse models depending on the reduction in its protein level.[10] This proves that some TSGs, like *PTEN*, are exquisitely sensitive to dose, while some, like *TP53*, are intermediately sensitive. Based on such observations, Berger et al has proposed a continuum model that accounts for subtle dosage effects of tumor suppressors including their regulation by microRNAs.[11] The dosage and function of haploinsufficient genes are critical, and understanding the impact of haploinsufficiency is important for assessment of interindividual genetic variation, as well as the molecular basis of haploinsufficiency disorders.

Haploinsufficiency of multiple genes cooperate to promote tumorigenesis, a phenomenon called 'compound haploinsufficiency'. The 5q deletion syndrome (5q-) is a paradigm of compound haploinsufficiency and demonstrates the importance of combinatorial interactions to elicit specific phenotypes.[12] Experimental evidence has shown that co-suppression of linked 8p TSGs promotes tumor formation more potently than any individual gene.[13] All the available evidence indicates that the functioning of a cell depends on the appropriate expression levels of proteins and, more importantly, that cell signalling pathways involve complex interactions between many proteins. Not only genes, but also microRNAs (miRNAs), a class of small non-coding RNAs, are shown to be haploinsufficient and to cause developmental abnormalities in humans.[14] In addition, genes involved in miRNA biosynthesis pathway, such as *DICER1*, *TARBP2* and *XPO5*, are identified as haploinsufficient tumor suppressors.[15–17]

miRNAs act at the post-transcriptional level of gene regulation through RNA-induced silencing complex (RISC) mediated translational inhibition or mRNA cleavage. The miRNA target recognition is mediated through the sequence complementarity between the 2–8 nt at the 5′ end of miRNA (seed sequence) and the 3′-UTR of target mRNA.[18] Based on the degree of complementarity, the mRNA is either guided for inhibition of translation or degradation resulting in the decrease of protein encoded by the target messenger.[19] This has extended the dimensions of haploinsufficiency as miRNAs can lead to the production of insufficient amount of proteins. Bioinformatics prediction and experimental analysis suggested that miRNAs can regulate approximately half of the mammalian genes, with a significant number of important oncogenes and tumor suppressor genes involved.[20,21] A recent genome-wide association study has suggested that a gene with more than two miRNA target sites will have higher variability of expression than a gene which is not regulated by a miRNA. The variability is further increased by SNPs in the miRNA target sites.[22] Polymorphisms in the miRNA regulatory pathway are a novel class of functional polymorphisms present in the human genome. The initial demonstration that miRNA binding site variations can result in a phenotype was provided by Abelson et al who identified a mutation in the miR-189 binding site of *SLITRK1* and its

association with Tourette's syndrome.[23] A pioneering study conducted by Carlo Croce's group showed that a germline mutation in pri-miR-16-1 resulted in low levels of miR-16-1 expression in familial chronic lymphocytic leukemia,[24,25] providing evidence that sequence variation in miRNA genes may affect function and result in cancer susceptibility. Pre- and pri-miRNA SNPs in miR-124-1, miR-146a, miR-196-a2, miR-218, miR-219-1, miR-26a-1, miR-27a, miR-423, miR-492, and miR-499 have already been shown to increase/decrease cancer risk in various populations.[26]

Single nucleotide polymorphisms (SNPs) are single base pair changes in DNA that occur with a frequency of about 1 in 12,500 bp in the genome.[27] Several studies have shown that SNPs in microRNA networks moderately increase the risk of cancer incidence.[28] In general, sequence variations in pri-miRNAs, pre-miRNAs, mature miRNAs, and microRNA binding sites potentially affects the processing and/or target selection of miRNAs. As a consequence, aberrant expression of hundreds of genes and pathways greatly affecting miRNA function may occur.[29] SNPs in mature miRNAs and miRNA binding sites function analogously to modulate the miRNA-mRNA interaction and create or destroy miRNA binding sites. Supporting this idea, SNPs within the miRNA binding sites of genes have been implicated in susceptibility to various types of cancer.[30–33] Additionally, functional support for individual miR-SNPs implicated in cancer do exist.

In this study we analyzed the role of SNPs that occur at miRNA binding sites and miR-SNPs and their contribution towards haploinsufficiency of tumor suppressor genes.

Materials and Methods

Datasets

A total of 110 haploinsufficient genes known to have a role in tumor initiation and progression were considered for the study. Among the total list, 63 tumorigenic haploinsufficient genes were obtained from Dang et al, 2008[34] while the remaining 47 genes were compiled using keyword search in PubMed and Online Mendelian Inheritance in Man (OMIM) database. For a comprehensive list of the haploinsufficient tumorigenic genes included in this study with their corresponding reference, see Supplement 1.

Analysis of 3′UTR polymorphisms in mRNA targets and miR seed

The haploinsufficient genes were analyzed for polymorphisms in their 3′UTR by submitting the respective gene ID in 'Polymorphism in microRNA Target Site' (PolymiRTS) database,[35] http://compbio.uthsc.edu/miRSNP/, designed to identify SNPs that disrupt the regulation of gene expression by miRNAs in human and mouse (last accessed on June 7th, 2012). The database is organized to provide links between SNPs in miRNA target sites, cis-acting expression quantitative trait loci (eQTLs), and the results of genome-wide association studies (GWAS) of human diseases. We applied filters to identify only the SNPs in the 3′UTR that were known to create binding sites for miRNAs and SNPs occurring in the seed region of miRNA themselves.

Classification and validation of SNPs obtained from polymiRTS

The SNPs predicted by the polymiRTS database were classified based on their effect into the following categories: (i) SNPs that lead to the creation of miRNA binding sites in the mRNA, (ii) SNPs that disrupted the originally existing miRNA binding sites and also resulted in creation of new miRNA binding sites in the mRNA, and (iii) miR-SNPs that altered the binding specificity of miRNAs. After classification, the SNPs were verified by submitting their rs ID in the NCBI's dbSNP Short genetic variation database http://www.ncbi.nlm.nih.gov/projects/SNP/ using the batch query option. The output was downloaded in BED format and analyzed. Among the total 158 SNPs submitted, 156 SNPs were validated by dbSNP while 2 miR-SNPs (rs116596918 and rs116838571) were found to be new entries and validated by microRNA-related Single Nucleotide Polymorphism database (http://www.bioguo.org/miRNASNP/miRNA_details.php).[36]

Functional network prediction using GeneMANIA Cytoscape plug-in

To identify the functional significance, GeneMANIA Cytoscape plug-in which employs the GeneMANIA

algorithm[37,38] was utilized with default settings to plot the interactions among the haploinsufficient TSGs. The complete list of tumorigenic haploinsufficient genes were uploaded to Cytoscape[39] using GeneMANIA plug-in (http://www.genemania.org/plugin/). Further, only the genes that were found to have SNPs in their miRNA binding sites and those that are non-specifically targeted by miRNAs harboring miR-SNPs were uploaded individually to study the interactions among this subgroup. The GeneMANIA Cytoscape plug-in integrates association networks from multiple sources into a single composite network using a conjugate gradient optimization algorithm. This algorithm produces networks from the data either directly (as in the case of protein or genetic interactions) or by using an in-house analysis pipeline to convert profiles to functional association networks.

Results and Discussion

Polymorphisms in 3′UTR of mRNA

Our analysis of tumorigenic haploinsufficient genes for genetic variations in the 3′UTR showed that 26% (29 out of 110) of them had at least one 3′UTR SNP resulting in creation of new binding sites for miRNAs thereby bringing the respective mRNA variant under the control of more new miRNAs. In total, 129 SNPs were found to create binding sites for 234 miRNAs in 29 genes (Table 1). Altogether, these SNPs contribute to haploinsufficiency by bringing the polymorphic mRNA under the control of more new microRNAs thereby leading to translational repression or mRNA degradation. *PPARA*, a nuclear transcription factor and *KIF1B*, a kinesin implicated in neuronal and non-neuronal tumors was found to harbor 17 SNPs creating putative binding sites for many miRNAs. On the contrary, the SNP rs121912664 in *TP53* creates putative miRNA binding site for miRNA-302 family, miR-520 family, miR-372, and miR-373-3p. The cell adhesion molecule *CDH1*, an important gene in tumor invasion with frequent allelic loss in metastatic tumors, was found to have 5 different SNPs that create putative binding sites for new miRNAs thereby altering the repertoire of miRNAs controlling this gene. Even *RB1*, the classical TSG, was predicted to have 3 SNPs that may down regulate its expression. If one allele is already lost in tumors, the remaining allele having at least one of these SNPs may be targeted by miRNAs and thereby lead to complete loss of cellular function, as postulated by Knudson's "two-hit hypothesis."

Another 10 SNPs were found to bring 8 different genes under the control of additional new miRNAs due to the presence of the respective SNP (Fig. 1). A single SNP can create miR binding site for several miRNAs as well as delete a miR binding site. For example, the SNP rs186304832 in the 3′UTR of *KIF1B* results in the loss of binding of 2 different microRNAs to the variant mRNA while 7 new microRNAs will be targeting this particular variant. This alters the miRNA mediated gene expression control resulting in aberrant regulation of expression. A gene that was previously under the control of one miRNA comes under the control of 3 different new miRNAs which leads to extensive translational repression resulting in protein haploinsufficiency as represented by the polymorphism rs1138533 in *CDH1*. This mechanism is totally new, since one allele is not lost due to mutation or deletion, as in the case of classical haploinsufficiency. Rather there is a decrease in the overall protein product. In homozygous state, this SNP may result in complete or partial reduction in the expression based on the ability of the repressing miRNA. However, in heterozygous state, the same SNP can lead to 50% or even subtler reduction in expression, which can explain 'quasi-insufficiency'.

Ours is the first study to identify the SNP and miRNA mediated control of tumor suppressor dose as well as highlighting the importance of genetic variations in cancer. This mechanism is in agreement with the continuum model for tumor suppression that is related to the level of expression or activity of the TSG rather than to the discrete step-by-step changes in gene copy number.[11]

Polymorphisms in microRNA seed sequence

In addition, SNPs that occur in the seed sequence of miRNAs can alter the binding specificity of these miRNAs resulting in the binding to new non-target mRNAs. We analyzed 19 such miR-SNPs that cause miRNAs to bind new haploinsufficient tumor suppressor genes (Table 2). The SNP rs4636784 occurring in the seed of hsa-miR-4305 alters the binding specificity, thereby enabling it to target 6 new

Table 1. List of SNPs in the haploinsufficient tumor suppressor genes that created microRNA binding sites in their 3′UTR.

Gene	Location	SNP ID	Allele change	microRNA binding site created for
ATM	108236469	rs3092834	C to T	hsa-miR-4252
	108237460	rs187308124	C to T	hsa-miR-4428
	108237837	rs227091	C to T	4 microRNAs*
	108238032	rs114847811	C to T	hsa-miR-600
	108239075	rs186644530	A to G	hsa-miR-2117
	108239099	rs191399133	T to C	hsa-miR-4448
	108239463	rs3092852	C to T	hsa-miR-1265
BAG1	33253621	rs191119067	G to C	hsa-miR-4452, hsa-miR-4680-3p
CAMTA1	7827003	rs183056496	G to A	hsa-miR-582-3p
	7828815	rs112733335	G to A	hsa-miR-342-3p
CDH1	68867609	rs35942505	C to T	6 microRNAs*
	68868146	rs140240766	C to A	hsa-miR-427
	68868576	rs9282654	T to G	hsa-miR-129-1-3p, hsa-miR-129-2-3p
	68869062	rs33967108	G to C	hsa-miR-1273a
	68869394	rs146556488	C to A	hsa-miR-369-3p
CDKN1B	12874056	rs114975362	G to T	hsa-miR-3919
CHEK1	125525308	rs148807246	A to G	hsa-miR-1184, hsa-miR-1205, hsa-miR-3158-5p
	125545904	rs192805943	G to A	hsa-miR-2964a-3p
CLU	27454684	rs9331949	A to G	hsa-miR-1283
CTCF	67672530	rs187258944	G to T	hsa-miR-2276
DFFB	3800550	rs146888429	G to A	hsa-miR-1227
	3800570	rs140704651	C to T	4 microRNAs*
DIRC2	122598847	rs150233566	G to C	hsa-miR-4797-5p
	122599452	rs180703954	A to C	hsa-miR-3927, hsa-miR-4451, hsa-miR-4732-5p
ETV6	12043995	rs183579990	C to G	hsa-miR-3160-3p, hsa-miR-558
	12044403	rs79663578	T to A	hsa-miR-5011-5p
	12045614	rs183305984	G to A	hsa-miR-3690
	12045861	rs72550771	T to C	hsa-miR-4774-5p
FBXW7	153243322	rs4399968	G to C	hsa-miR-338-3p, hsa-miR-634
FEN1	61564571	rs141406755	T to C	hsa-miR-3654
KIF1B	10365509	rs180831292	C to G	hsa-miR-4768-5p
	10365953	rs72640311	T to G	hsa-miR-1972
	10366284	rs115354754	A to C	hsa-miR-3934, hsa-miR-764
	10366763	rs56164683	A to G	hsa-miR-27a-3p, hsa-miR-27b-3p
	10366766	rs187140427	A to T	hsa-miR-376c
	10367056	rs41274464	G to A	hsa-miR-148b-5p, hsa-miR-3168
	10367348	rs142468272	C to A	4 microRNAs*
	10367422	rs2004034	G to A	6 microRNAs*
	10367577	rs143823495	G to T	hsa-miR-4494, hsa-miR-4499
	10367816	rs145128126	A to T	hsa-miR-3941, hsa-miR-466, hsa-miR-4672
	10437247	rs2155760	C to T	8 microRNAs*
	10437802	rs4240913	G to A	hsa-miR-645
	10438336	rs189264558	A to G	hsa-miR-516a-3p, hsa-miR-516b-3p
	10439362	rs186201500	A to C	hsa-miR-199a-5p, hsa-miR-199b-5p, hsa-miR-644a
	10439538	rs184232081	G to A	hsa-miR-4425
	10440756	rs192312673	C to T	hsa-miR-4797-5p
	10440757	rs148005450	G to A	hsa-miR-4534
KLF5	73650341	rs117651109	C to T	hsa-miR-5579-5p
MDM2	69235650	rs144972734	A to G	hsa-miR-671-5p
	69236143	rs190072365	C to T	hsa-miR-934
	69236269	rs142063414	G to A	hsa-miR-3198, hsa-miR-4309
	69236548	rs1690917	G to T	4 microRNAs*
	69237299	rs183077346	T to A	hsa-miR-889
	69237446	rs150137388	A to G	hsa-miR-9-3p

(*Continued*)

Table 1. (*Continued*)

Gene	Location	SNP ID	Allele change	microRNA binding site created for
	69238249	rs141938174	C to T	hsa-miR-4705
	69238568	rs148584832	G to A	hsa-miR-4522
	69238940	rs184278637	G to A	6 microRNAs
PAX5	36839029	rs184505592	G to C	hsa-miR-4520a-3p
PPARA	46631658	rs4253801	A to G	hsa-miR-4709-5p
	46631718	rs183307497	A to G	hsa-miR-5095, hsa-miR-600
	46631838	rs188241700	T to G	hsa-miR-1470, hsa-miR-4287, hsa-miR-4685-3p
	46632587	rs9615264	G to A	hsa-miR-1231
	46632932	rs181140325	T to A	hsa-miR-4277
	46633120	rs78922364	G to C	hsa-miR-499b-5p
	46633549	rs115356860	C to T	hsa-miR-892b
	46633564	rs148478205	C to T	hsa-miR-4252
	46634352	rs184920139	G to A	hsa-miR-301a-5p
	46634514	rs41418950	C to T	hsa-miR-936
	46635061	rs41403648	G to A	hsa-miR-3944-5p, hsa-miR-5094
	46636623	rs56180992	G to A	hsa-miR-515-5p, hsa-miR-519e-5p
	46637760	rs181781384	A to G	hsa-miR-4733-3p
	46638155	rs1055658	G to C	hsa-miR-744-5p
	46638209	rs79700435	G to A	hsa-miR-3191-5p, hsa-miR-518c-5p
	46639161	rs187605878	A to G	hsa-miR-3157-3p
	46639611	rs117317496	G to C	hsa-miR-5584-5p
PTCH1	98205709	rs28413010	A to T	hsa-miR-126-5p, hsa-miR-4795-3p
	98206995	rs41316950	C to G	hsa-miR-4690-3p, hsa-miR-5685
PTEN	89725452	rs3895069	T to G	hsa-miR-5003-5p
	89726622	rs79395513	C to A	hsa-miR-651
	89726726	rs180953647	C to T	hsa-miR-510
RAD50	131980172	rs146790944	A to G	hsa-miR-300, hsa-miR-381
	131980267	rs139468574	A to G	hsa-miR-4524a-5p, hsa-miR-4524b-5p
	131980270	rs75939007	A to G	4 microRNAs*
RB1	49054521	rs187845274	T to A	hsa-miR-4638-3p
	49054779	rs150194261	T to C	hsa-miR-145-5p, hsa-miR-5195-3p
	49055241	rs192758219	G to T	hsa-miR-1323, hsa-miR-548o-3p
RXRA	137328831	rs1805346	G to A	hsa-miR-3688-3p
	137328936	rs189590058	C to T	hsa-miR-4738-3p
	137329304	rs4240711	G to A	hsa-miR-3184-3p
	137329411	rs34449651	G to C	hsa-miR-3919
	137329652	rs35283205	G to C	hsa-miR-4685-5p
	137330306	rs180869938	T to C	hsa-miR-1908, hsa-miR-663a
	137330727	rs17847916	C to T	hsa-miR-4721
	137330802	rs10119893	G to A	5 microRNAs*
	137330980	rs139882385	T to C	hsa-miR-518a-5p, hsa-miR-527
	137331443	rs139749844	G to A	hsa-miR-3170, hsa-miR-5572
	137331999	rs11103561	G to A	hsa-miR-4722-3p, hsa-miR-4769-3p
	137332362	rs187745805	G to A	hsa-miR-5010-3p, hsa-miR-599
	137332378	rs137912651	G to C	hsa-miR-301a-5p
SERPINB5	61171674	rs112795011	A to C	hsa-miR-4535, hsa-miR-4776-5p
SMAD4	48605247	rs28403611	A to G	hsa-miR-552
	48605297	rs191822473	G to A	hsa-miR-656
	48605643	rs183984903	A to G	hsa-miR-5095
	48607107	rs189547031	G to C	hsa-miR-5584-5p
	48607497	rs191637734	A to T	hsa-miR-421, hsa-miR-4272
	48608123	rs191317135	C to T	hsa-miR-3163
	48609056	rs150556996	G to T	hsa-miR-4496
	48609588	rs112739300	C to T	hsa-miR-297, hsa-miR-3149, hsa-miR-675-3p

(*Continued*)

Table 1. (*Continued*)

Gene	Location	SNP ID	Allele change	microRNA binding site created for
	48609716	rs184516281	T to C	hsa-miR-584-3p
	48609918	rs145596898	G to A	hsa-miR-4694-3p
	48610254	rs146551171	T to C	23 microRNAs*
	48610822	rs12954419	T to A	hsa-miR-5696
	48610844	rs181250637	G to C	hsa-miR-636
	48611357	rs183472455	C to T	hsa-miR-371b-5p, hsa-miR-373-5p, hsa-miR-616-5p
SOX9	70121452	rs74999341	T to C	hsa-miR-4759
	70121525	rs185467299	C to T	hsa-miR-4514, hsa-miR-4692, hsa-miR-4742-5p
TNFAIP3	138202555	rs140042278	G to T	hsa-miR-208a, hsa-miR-208b, hsa-miR-499a-5p
	138202864	rs76710415	A to C	hsa-miR-4533
	138203096	rs73778206	T to C	hsa-miR-223-3p
	138203386	rs191520762	T to G	hsa-miR-4772-3p
	138203884	rs73778207	G to A	hsa-miR-96-3p
TP53	7572039	rs35659787	G to A	hsa-miR-516b-5p, hsa-miR-518c-5p
	7574010	rs17882252	G to A	hsa-miR-127-5p
	7574015	rs121912664	G to A	12 microRNAs*
VHL	10191771	rs34362094	C to T	hsa-miR-5579-5p
	10192172	rs190102073	A to G	hsa-miR-4448
	10193434	rs115303528	G to A	hsa-miR-592, hsa-miR-599
	10193848	rs145666034	G to A	hsa-miR-3545-3p
	10194195	rs187719061	A to G	hsa-miR-371a-5p

Notes: This table enlists the 129 SNPs that create new miRNA binding sites in the 3′UTR of their respective haploinsufficient tumor suppressor gene. Table compiled from the data provided by PolymiRTS database. *For convenience these microRNAs are not indicated in the table, and for details the reader is referred to Supplement 2.

haploinsufficient genes (*CDH1, KIF1B, SMAD4, DFFB, FOXP1*, and *PTEN*). This can contribute to compound haploinsufficiency, wherein multiple genes cooperate to cause a particular phenotype.[7,13] Alteration in one miRNA can alter the entire set of genes under its control thereby providing an advantage for cancer initiation and progression. In addition, the acquisition of miR-SNPs in tumors is simple enough to deregulate many genes than the need for individual mutations in all of them. This mechanism of miR-SNP mediated down regulation can also be extended to the unexplained allelic loss of *KIF1B* in neuroblastomas, where the tumor suppressor was shown to have no mutations or promoter methylation.[40] Tumors not showing genetic alterations in the TSG, but with aberrant

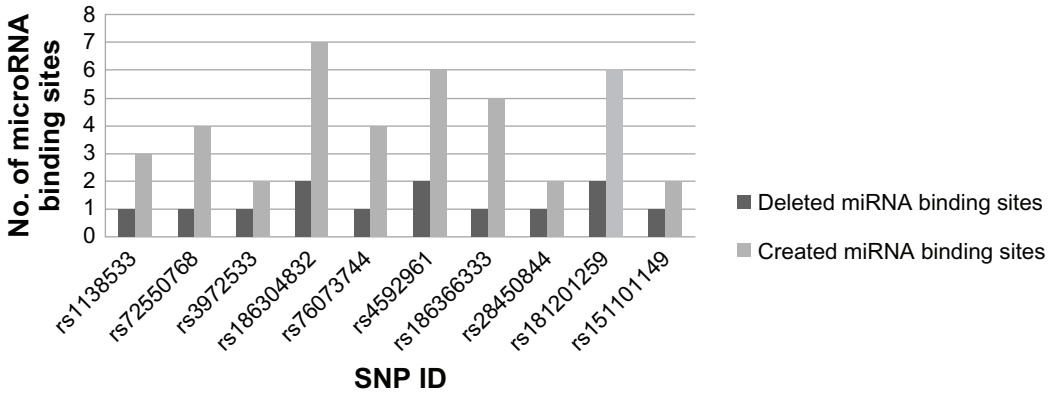

Figure 1. Effect of SNPs occurring in the 3′UTR of haploinsufficient tumor suppressor genes.
Notes: SNPs in the 3′UTR of haploinsufficient tumor suppressor genes result in creation of new miRNA binding sites as well as deletion of originally existing miRNA binding sites. The 10 SNPs plotted here against their effect on miRNA binding sites contributes to haploinsufficiency, as they bring the polymorphic mRNA under the control of more new microRNAs.

Table 2. SNPs in miR seed and their new target haploinsufficient genes.

miR ID	SNP ID	miR seed	Allele	Haploinsufficient gene targets
hsa-mir-125a	rs12975333	CCCUGA[G/U]	G/T	ATM, MSH2
hsa-miR-1268	rs28599926	GGGC[G/A]UG	G/A	ATM, PAX5, PPARA, PTEN, NBN
hsa-miR-1469	rs116596918	UCGGC[G/C]C	C/G	DDB2, FOXP1
hsa-miR-3610	rs112072631	AAUCGG[A/C]	C/A	SERPINB5, SMAD4
hsa-miR-3615	rs112977728	CU[C/U]UCGG	C/T	RAD50, NBN, TP53BP2
hsa-miR-3618	rs12159555	GU[C/G]UACA	C/G	DFFB, PPARA, SMAD4, FOXP1
hsa-miR-3689b	rs116838571	GUGAU[A/U]U	T/A	CDH1, CDKN1B, FBXW7, RB1, VHL, BRCA2, ROR2, TP53BP2, YWHAE
hsa-miR-379	rs61991156	GGUAG[A/G]C	A/G	CAMTA1, DFFB, TNFAIP3, VHL
hsa-miR-4257	rs74743733	CAGA[G/A]GU	A/G	CHEK1, DIRC2, ETV6, PAX5, SOX9
hsa-miR-4284	rs11973069	GGCUCA[C/U]	C/T	BAG1, ETV6, TP53BP2
hsa-miR-4305	rs4636784	CUA[G/C]ACA	G/C	CDH1, DFFB, FBXW7, KIF1B, PTEN, SMAD4, FOXP1, NBN, MDM2
hsa-miR-4322	rs114399468	UGUGG[G/A]C	A/G	CHEK1, KIF1B, PTEN, RAD50, SERPINB5
hsa-miR-557	rs78825966	UUUGCA[C/U]	C/T	BAG1, CHEK1, BRCA2, RPL37A, KLF5
hsa-miR-585	rs62376935	GG[G/A]CGUA	G/A	BAG1, KIF1B, NBN, TP53BP2
hsa-miR-605	rs113212828	[A/G]AAUCCC	A/G	RAD50, RB1, VHL, MSH2
hsa-miR-642a	rs78902025	UCCC[U/G]CU	G/T	ATM, PPARA, SERPINB5
hsa-miR-642b	rs111664333	GA[C/U]ACAU	T/C	ATM, RAD50, SMAD4, NBN, TP53BP2
hsa-miR-936	rs79924817	C[A/G]GUAGA	G/A	ATM, BAG1, PPARA, RAD50, XPO5
hsa-miR-938	rs12416605	[G/A]CCCUUA	G/A	BAG1, PPARA, PTEN, SMAD4, SOX9, TNFAIP3, XPO5, MDM2

Notes: This table represents the miR-SNPs that render microRNAs to bind with non-specificity to haploinsufficient tumor suppressor genes. Table compiled from the data extracted from PolymiRTS database.

TSG levels due to miRNA misregulation, could behave like tumors with deletion or mutation of the gene. Importantly, mapping the interactions between miRNAs and TSGs could be useful for defining and predicting cancer susceptibility and therapeutic response.

Networks

Although reports on haploinsufficient genes associated with cancer is limited, analysis of the existing data shows that at least one third of total haploinsufficient genes to be tumorigenic. Tumor suppressors often act as components of complex networks, the overall function of which can be impaired by genetic and epigenetic alterations.[41,42] For this reason we analyzed whether the haploinsufficient tumor suppressor genes share a common pathway or fall in a common interacting or functional network. The GeneMANIA algorithm was utilized for this purpose, and results indicate that almost 99% of haploinsufficient genes have a strong interaction, either directly or indirectly. *SMAD5* was excluded from the list as it was not recognized by the algorithm. Co-expression, co-localization, physical and genetic interactions, pathway interactions, and other interactions that arise through shared structural domains were shown to exist between the queried genes (Fig. 2). *SPRED1* was the only gene to fall out of this network however co-expression links it to the group. Apart from the queried list of genes, the algorithm identified several other genes to be a part of this network including *DROSHA, DFFA, MRE11A, TRAPPC2L, TERF2, CDK6, BUB1, WRN, CCNA2,* and *RAD51*. Interestingly, the haploinsufficient status of some of these genes has already been described in developmental abnormalities and other diseases,[43–45] while some others are shown to have a role in tumor formation and their haploinsufficient status is yet to be established.[46]

Next we tested whether the 39 genes with SNPs in miRNA binding sites or targeted by miRNAs with altered specificity due to miR-SNPs fall in to a particular network or pathway. Results of GeneMANIA networking analysis suggested strong interactions between these genes (Fig. 3). DNA repair genes and cell cycle checkpoint genes

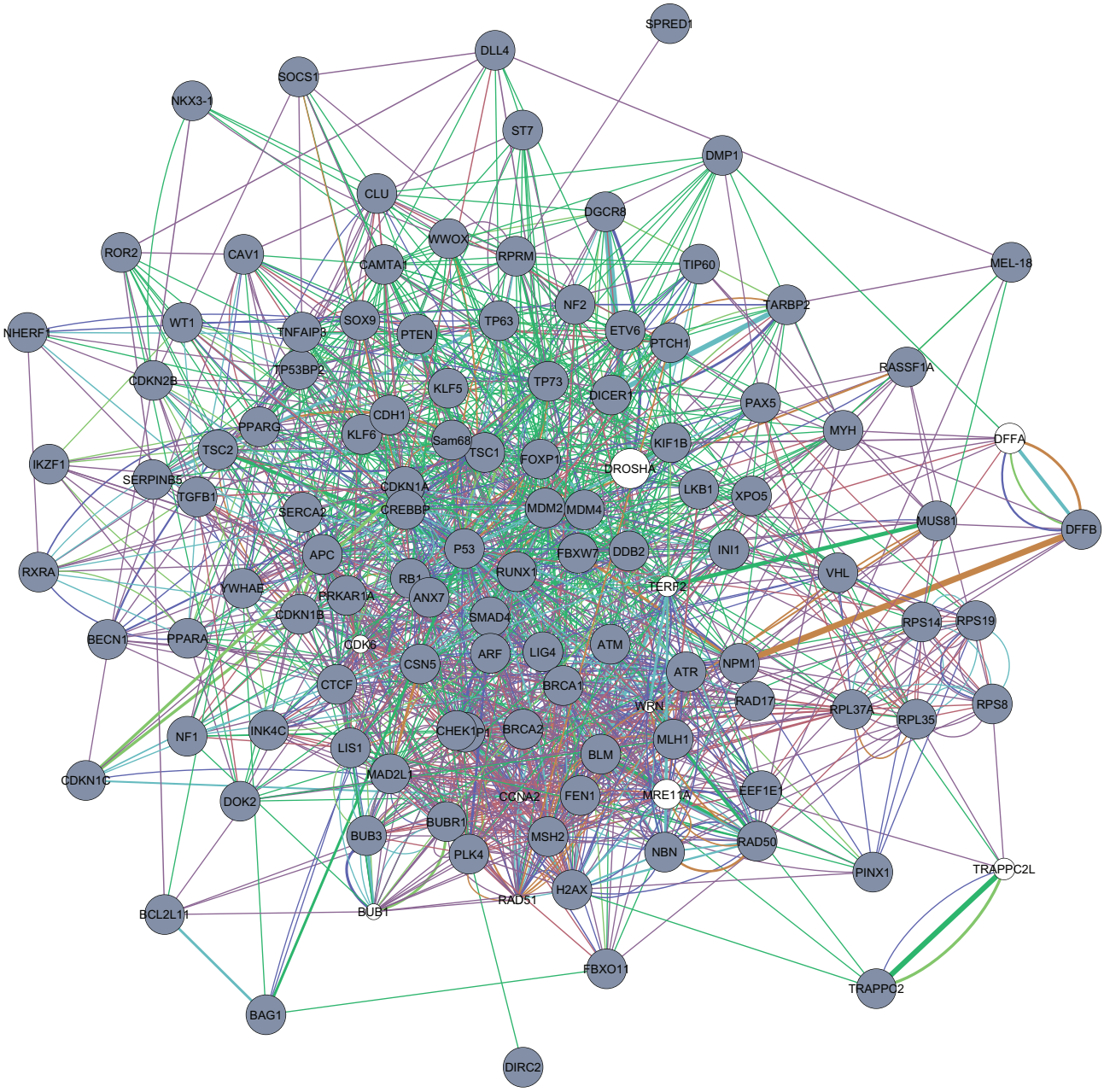

Figure 2. Interactions among 109 haploinsufficient genes plotted using GeneMANIA plug-in available in Cytoscape.
Notes: Networking analysis of 109 genes by GeneMANIA algorithm revealed different type of interactions between them. Gray circles represent the queried genes, and white circles represent the predicted genes that mediate interactions. Color representations for edges: purple—co-expression, dark blue—physical interaction, green—genetic interaction, light blue—co-localization, and brown—predicted. The network consists of physical, genetic and predicted interactions which can be clearly seen.

were enriched in this network and any aberrations in their expression offers selective advantage for the tumors to acquire additional genomic changes or mutations. *DFFB* was linked by co-expression, while *DIRC2* was shown to be completely out of the group. The following genes were found to be a part of this network and to mediate interactions between the queried genes: *MRE11A, DFFA, CCNB1, POLQ, AP1B1, RAD51, CCNG1, NPM1, DDB*1, and *WRN*. The identification of the mRNA targets that mediate the actions of miRNAs in disease pathways can reveal previously unrecognized components that may serve as targets for more traditional drug development.

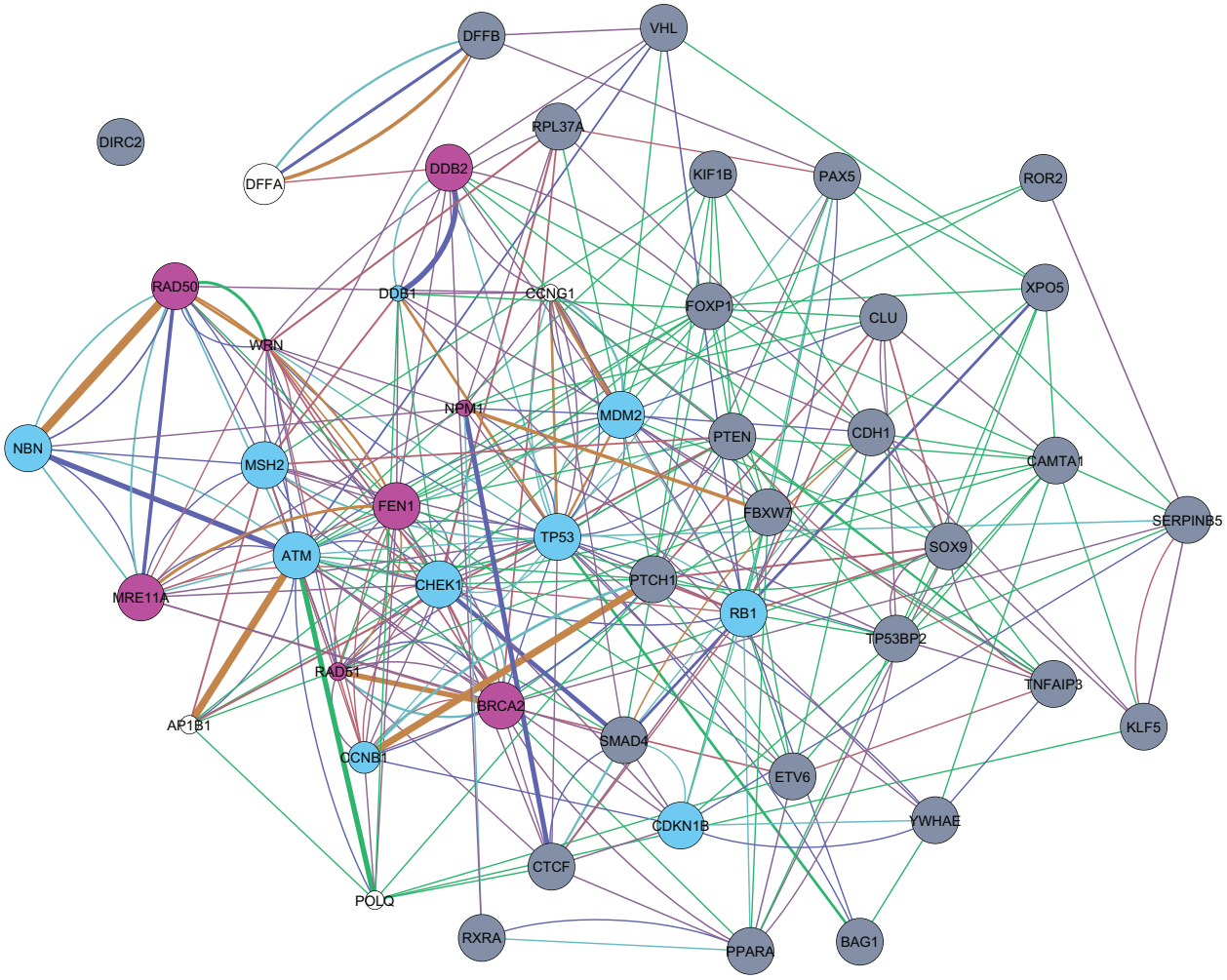

Figure 3. Interactions among 39 haploinsufficient genes that either had miRNA binding site SNPs or were targeted non-specifically by miRNAs carrying miR-SNPs.
Notes: Networking analysis of 39 genes by GeneMANIA algorithm revealed different type of interactions among them. Gray circles represent the queried genes and white circles represent the predicted genes that mediate interactions. Pink circles denote the genes involved in DNA repair and blue circles represent the cell cycle checkpoint genes. Among the queried list, *DIRC2* does not show any type of interaction. Color representations for edges: purple—co-expression, dark blue—physical interaction, green—genetic interaction, light blue—co-localization, and brown—predicted. The network consists of physical, genetic and predicted interactions which can be clearly seen.

Conclusion

With the expanding understanding of the roles of miRNAs in various cellular processes and diseases, this is the first report to discuss the role of miRNAs and its contribution towards tumor suppressor gene haploinsufficiency at the protein level via 3′UTR SNPs and miR-SNPs. Further, this could be an alternate means for attaining compound heterozygosity observed in several tumors and the mechanism is in complete agreement with the continuum model of tumor suppression.[11] Our data suggests that 26% of the tumorigenic haploinsufficient genes were brought under the control of new miRNAs due to 3′UTR SNPs. The identification of 10 SNPs that drive haploinsufficiency by bringing the polymorphic mRNA under the control of miRNAs, other than the miRNAs which are deleted, needs experimental validation in tumors. This alteration in the miRNA mediated gene regulation may cause predisposition to cancer initiation and progression.

Evidence for co-operative contribution of oncogenic mutations with tumor suppressor haploinsufficiency also exist.[47] The realization that miR-SNPs play central roles in the aberrant regulation of tumor suppressor genes has provided a new perspective on our understanding of pathophysiologic mechanisms. In addition, networking analysis reveals strong interactions between the haploinsufficient

tumor suppressor genes. Any subtle alteration in this network of genes due to SNPs at the 3'UTR and miR-SNPs may contribute to pathogenesis. We suggest that at least a few among these SNPs and their effect on miRNA binding will aid in the diagnosis and/or prognosis of such type of cancers, if experimentally validated. While challenges remain in this regard, the pace of development in this field suggests that new discoveries are forthcoming.

Our approach has currently focused only on analyzing 3′UTRs, although a small subset of miRNAs can target 5′UTRs, coding regions, and gene promoters. miR-SNPs leading to non-specific binding of miRNAs to tumor suppressors may also result in the loss of binding to their original targets. If the target is an oncogene, it will be over expressed and will result in tumorigenesis. This has not been focused on in this study. Since the rules for miRNA binding to its target changes constantly,[48] the databases need constant updating. The 110 tumor suppressor genes chosen for our study were already proven to be haploinsufficient in several cancers through experimental evidence. This list may likely grow due to continuous identification of haploinsufficient tumor suppressors.

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Author Contributions

AKM conceived and designed the study. MM and GR performed the experiments. AKM, MM, and GR analyzed the data. MM and AKM prepared the manuscript. All authors read and approved the final manuscript.

Competing Interests

Author(s) disclose no potential conflicts of interest.

Disclosures and Ethics

As a requirement of publication author(s) have provided to the publisher signed confirmation of compliance with legal and ethical obligations including but not limited to the following: authorship and contributorship, conflicts of interest, privacy and confidentiality and (where applicable) protection of human and animal research subjects. The authors have read and confirmed their agreement with the ICMJE authorship and conflict of interest criteria. The authors have also confirmed that this article is unique and not under consideration or published in any other publication, and that they have permission from rights holders to reproduce any copyrighted material. Any disclosures are made in this section. The external blind peer reviewers report no conflicts of interest.

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Supplement I. List of Haploinsufficient genes known to be involved in tumorigenesis.

Haploinsufficient genes	References
ANX7, APC, ARF, ATM, BAG1, BECN1, BRCA1, BRCA2, BUBR1, CAMTA1, CAV1, CDKN1B, CDKN1C, CDKN2B, CSN5, CTCF, DFFB, DIRC2, EEF1E1, ETV6, FBXW7, FOXP1, H2 AX, INI1, INK4C, KLF6, LIG4, LIS1, LKB1, MEL-18, MYH, NHERF1, NKX3–1, NPM1, P53, PAX5, PLK4, PPARA, PPARG, PRKAR1 A, PTCH1, PTEN, RAD50, RASSF1 A, ROR2, RPRM, SAM68, SERCA2, SMAD5, SOCS1, SOX9, SPRED1, ST7, TGFB1, TP53BP2, TP73, TRAPPC2, TSC1, TSC2, VHL, WT1, WWOX, YWHAE	11
ATR	18
BIM (BCL2 L11)	17
BLM	21
BUB3	30
CDH1	44
CDKN1 A	28
CHK1 (CHEK1)	33
CLU	9
CREBBP (CBP)	49, 47, 5
DDB2	26
DGCR8	42
DICER1	32, 2, 41
DLL4	19, 23
DMP1	25, 51, 34
DOK2	4
FBXO11	14
FEN1	31
IKZF1 (IKAROS)	39, 29, 52
KIF1Bβ	40
KLF5	10
MAD2 L1	38
MDM2 and MDM4	50
MLH1	48
MSH2	12, 13, 6
MUS81	36
NBN	15
NF1	27, 59
NF2	35
PINX1	58
RAD17	8
RB1	53, 57
Ribosomal proteins (RPL35, RPL37 A, RPS19 and RPS8)	1
RPS14	16, 3
RUNX1	45
RXRA	24
SERPINB5 (Maspin)	43
SMAD4/DPC4	56
TARBP2	37
TIP60	20
TNFAIP3	55, 7
TP53BP1	54
TP63 (TAp63)	46
XPO5	22

Supplement 2. Supporting information for Table 1.

Gene	Location	SNP ID	Allele change	miR ID
ATM	108237837	rs227091	C to T	hsa-miR-3664-3p, hsa-miR-4433-3p, hsa-miR-4768-3p, hsa-miR-512-5p
CDH1	68867609	rs35942505	C to T	hsa-miR-548ae, hsa-miR-548ah-3p, hsa-miR-548aj-3p, hsa-miR-548am-3p, hsa-miR-548aq-3p, hsa-miR-548x-3p
DFFB	3800570	rs140704651	C to T	hsa-miR-3664-3p, hsa-miR-4433-3p, hsa-miR-4768-3p, hsa-miR-512-5p
KIF1B	10367348	rs142468272	C to A	hsa-miR-4435, hsa-miR-4701-5p, hsa-miR-548s, hsa-miR-588
	10367422	rs2004034	G to A	hsa-miR-25-3p, hsa-miR-32-5p, hsa-miR-363-3p, hsa-miR-367-3p, hsa-miR-92a-3p, hsa-miR-92b-3p
	10437247	rs2155760	C to T	hsa-miR-125a-5p, hsa-miR-125b-5p, hsa-miR-4319, hsa-miR-4446-5p, hsa-miR-4732-3p, hsa-miR-4755-5p, hsa-miR-5006-3p, hsa-miR-670
MDM2	69236548	rs1690917	G to T	hsa-miR-548ac, hsa-miR-548d-3p, hsa-miR-548h-3p, hsa-miR-548z
	69238940	rs184278637	G to A	hsa-miR-3609, hsa-miR-4796-3p, hsa-miR-519a-3p, hsa-miR-519b-3p, hsa-miR-519c-3p, hsa-miR-548ah-5p
RAD50	131980270	rs75939007	A to G	hsa-miR-199a-3p, hsa-miR-199b-3p, hsa-miR-3129-5p, hsa-miR-936
RXRA	137330802	rs10119893	G to A	hsa-miR-1254, hsa-miR-1271-3p, hsa-miR-3116, hsa-miR-550a-3-5p, hsa-miR-550a-5p
SMAD4	48610254	rs146551171	T to C	hsa-miR-142-5p, hsa-miR-548a-5p, hsa-miR-548ab, hsa-miR-548ak, hsa-miR-548am-5p, hsa-miR-548ap-5p, hsa-miR-548aq-5p, hsa-miR-548ar-5p, hsa-miR-548as-5p, hsa-miR-548au-5p, hsa-miR-548av-5p, hsa-miR-548b-5p, hsa-miR-548c-5p, hsa-miR-548d-5p, hsa-miR-548h-5p, hsa-miR-548i, hsa-miR-548j, hsa-miR-548k, hsa-miR-548o-5p, hsa-miR-548w, hsa-miR-548y, hsa-miR-559, hsa-miR-5590-3p
TP53	7574015	rs121912664	G to A	hsa-miR-302a-3p, hsa-miR-302b-3p, hsa-miR-302c-3p, hsa-miR-302d-3p, hsa-miR-302e, hsa-miR-372, hsa-miR-373-3p, hsa-miR-520a-3p, hsa-miR-520b, hsa-miR-520c-3p, hsa-miR-520d-3p, hsa-miR-520e

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