Is the Identification of SNP-miRNA Interactions Supporting the Prediction of Human Lymphocyte Transcriptional Radiation Responses?

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Abstract: Genome-Wide Association Studies (GWAS) are of great importance in identifying the genetic variants associated with traits/diseases. Due to the high number of candidate SNPs some filtering techniques are necessary to be applied. The aim of the study was to develop the comprehensive approach allowing for detailed analysis of both SNP-gene and SNP-miRNA-gene relations. We elaborated and optimized the novel signal analysis pipeline improving significantly the results of the analysis on genotype-phenotype interplay. Direct links between genotype results and gene expression levels were enriched by detailed analysis of SNP-miRNA-gene interactions at both mature miRNA structure/seed region and target binding site level. The proposed technique was applied to the data on lymphocyte radiation response and increased by almost 100% number of potential functional SNPs.

1 INTRODUCTION

Genome-Wide Association Studies (GWAS) are the most popular kind of research to identify the genetic variants associated with traits/diseases. Methods to identify the candidate single nucleotide polymorphisms (SNPs) depend on the study design and different statistical approaches and have been widely discussed in (Bush and Moore, 2012; Evangelou and Ioannidis, 2013). Filtering the obtained set of candidate SNPs by applying the biological information, such as the potential effects of mutation in gene coding regions, modifications of gene related signal pathways or gene ontology terms, or the detailed analysis of microRNA-SNP interactions can reduce the number of false candidate SNPs, and become a separate research field in GWAS analysis. Over the years, a lot of algorithms were proposed to support such functional analysis in (Patnala et al., 2013; Wang et al., 2010), but the most challenging issue still belongs to the prediction of microRNA-SNP interactions, as the understanding of microRNAs function continue to increase.

MicroRNAs (miRNAs) are small non-coding RNAs, which regulate gene expression. Single nucleotide polymorphisms may be functional with respect to miRNAs biogenesis or the specific roles of mature miRNA (Dong et al., 2013). From the miRNAs biogenesis pathway, SNPs can modify the primary miRNA (pre-miRNA) or mature miRNA structures. While considering the regulatory function of miRNA, specifically relevant target site recognition of the seed region of miRNA (6-8 nucleotides at the 5' end of the miRNA) is of great importance. Roughly, single nucleotide polymorphisms can impact the functionality of miRNA by modifying its primary target binding sites or by creating new binding sites. As a consequence gene disregulation may lead to phenotype changes and eventually prove to be critical for the susceptibility to cancer as well as other diseases (Slaby et al., 2012; Vitale et al., 2011). Although some of the published works include miRNA-SNP interaction analysis, most of them focus on SNPs in primary binding site only (Deveci et al., 2014).

Radiation sensitivity (radiosensitivity), which is the relative susceptibility of cells, tissues, organs or

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organisms to the harmful effect of radiation, can be influenced by many factors amongst which are epigenetic modifications (Ma et al., 2010; Lahtz and Pfeifer, 2011), and miRNA regulation (Zhao et al., 2012). Among the group of radiation-responsive genes, BBC3 (BCL2 binding component 3) is of great importance, since it encodes a protein named PUMA (p53 upregulated modulator of apoptosis) which is involved in p53-dependent and -independent apoptosis induced by a variety of signals amongst which there is ionizing radiation (Yu and Zhang, 2005). This gene is known as a reliable biomarker of radiation exposure (Budworth et al., 2012). It is a good candidate to investigate the potential role of SNP-gene and SNP-miRNA interactions in radiosensitivity and on the long term, relevant for a better understanding of inter-individual radiation responsiveness supposed to be linked at least partially to apoptosis mechanisms. This could lead to radiotherapy regime improvements personalized cancer treatments.

In this study we perform a novel comprehensive functional analysis taking into consideration both SNP-gene and SNP-miRNA interactions. We demonstrate that this type of approach can potentially improve the discovery of candidate process relevant SNPs compared to standard SNP-gene based methods only.

2 MATERIALS AND METHODS

2.1 Materials

The group under investigation is composed of 44 unrelated Caucasian individuals (unR), with two types of data collected.

The first dataset includes qPCR measurements for BBC3 gene, taken in two conditions: 1) in normal conditions - no irradiation, and 2) just after the irradiation with a single dose of 2Gy. The irradiation was performed at room temperature with an A.G.O. HS X-ray system (Aldermaston, Reading, UK) (output 13 mA, 250 kV peak, 0.5 Gy/min for doses 0.5 4 Gy and 0.2 mA 4.9 mGy/min for doses up to 100 mGy). The T-lymphocyte cultures were used and prepared using the method described previously (O'Donovan et al., 1995; Finnon et al., 2008).

The second dataset includes results on genotyping 567,096 polymorphisms (SNP) by Axiom GW Human hg36.1 arrays. The BBC3 gene expressions obtained at both experimental conditions were previously published (Kabacik et al., 2011a; Kabacik et al., 2011b; Manning et al., 2013).

2.2 Selection of Polymorphisms

At the first step, the quality control for both qPCR and microarray experiments was performed. During the next step, the genotype-phenotype interactions were modelled per every SNP following the procedure previously presented in (Zyla et al., 2014). The genotype-phenotype models were constructed for both BBC3 expression level in normal condition (no irradiation, 0Gy) and for standardized fold change (FCH) signal. The final two sets of candidate radiosensitivity related SNPs were defined as follows: 1) all SNPs significantly (p-value <0.05) related to the BBC3 gene expression fold change (FCH) and not significantly related to the BBC3 gene expression level in normal condition (0Gy) - named SET 1, and 2) all SNPs significantly related to BBC3 gene expression fold change (FCH) and significantly related to the BBC3 gene expression level in normal condition (0Gy) - SET 2 (Figure 1).

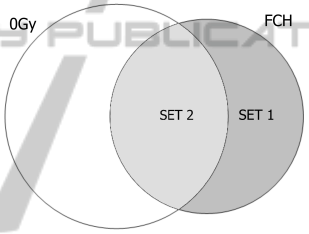

Figure 1: The definition of the final two sets of candidate radiosensitivity related SNPs: 1) SET 1 - all SNPs significantly related to the expression fold change (FCH) and not significantly related to the expression in normal condition (0Gy) - dark grey colour; 2) SET 2 - all SNPs significantly related to the expression fold change (FCH) and significantly related to the expression in normal condition (0Gy) - light grey colour.

2.3 Functional Analysis

2.3.1 SNP-gene Interactions

The obtained two sets of candidate radiosensitivity related SNPs were limited to the SNPs occurring in genes. The information on SNP location in genome and its transcriptomic assignment was collected using SNPLab software (GRCh38). Each SNP located in exon and having missense activity (nonsynonymous SNP - nsSNPs) was further analysed to predict its impact on cell functioning. Polymorphisms of this type lead to a change of the amino acid in the protein sequence. To assess the impact of nsSNPs on the organism the PredictSNP software was used (Bendl et al., 2014), which integrates the results from six the most popular algorithms (MAPP, SIFT SNAP,

PolyPhen1, PolyPhen2, and PhD-SNP). Additionally, each of the genes modified by candidate SNPs was investigated toward the overrepresentation of Gene Ontology (GO) terms (Szkiba et al., 2014), and KEGG pathways (Beissbarth and Speed, 2004).

2.3.2 SNP-miRNA interactions - standard approach

Three the most popular bioinformatical systems which analyse miRNA and SNP interactions are: 1) SNPinfo (Xu and Taylor, 2009), 2) PolymiRTS Database (Bhattacharya et al., 2014), and 3) miRSNP (Liu et al., 2012). All of the above focus mainly on SNP and miRNA binding site interactions, in particular SNPinfo focuses on polymorphisms in 3' UTR region of mRNA and uses miRanda algorithm (Griffiths-Jones et al., 2008) for target binding site prediction, while PolymiRTS Database searches for SNPs modifying seed regions of miRNA and uses TargetScan algorithm (Lewis et al., 2005) and experimental data (Dweep et al., 2014; Hsu et al., 2014; Vergoulis et al., 2012) for target binding site predictions. The last of the mentioned algorithms, miRSNP, as the only one identifies polymorphisms in pre-miRNA sequences and uses miRanda algorithm for identification of SNPs modifying the binding sites.

2.3.3 Comprehensive SNP-miRNA Analysis Procedure

The detailed up-to-date analysis of SNP interaction on mature miRNA or miRNA's seed region done together with tracking of binding site modifications seem to be crucial for our study. Since none of the above systems allows for complete analysis of SNPs and mature miRNA structure interactions, we have developed a software performing the analyses. Using miRBase v21 we found chromosomal coordinates (GRCh38) of mature miRNAs and miRNAs seed regions and compared them with coordinates of analysed SNPs from previously found SET 1 or SET 2. Combining that set with miRNAs having binding sites modified by SNPs from the same SET 1 or SET 2 gives the complete set of miRNAs under investigation allowing for the detection of candidate SNPs responsible for modification of miRNA gene expression regulation processes. The next step required the definition of binding sites being targets for chosen miRNAs, and it was done at two levels: experimentally validated sites and in silico predicted sites only. We used miRTarBase (Hsu et al., 2014) and miRWalk (Dweep et al., 2014) to find experimentally validated targets and for every miRNA which does not have validated target genes we used TargetScan and DIANA-microT-CDS prediction algorithms. As it has become a common practice for researches to look at predictions produces by several miRNA-target prediction programs, we focused on intersection of results obtained from this two algorithms. The group of genes being targets for chosen miRNAs was investigated toward the overrepresentation of Gene Ontology (GO) terms and KEGG pathways (Beissbarth and Speed, 2004). The data flow pipeline for whole procedure is presented in Figure 2.

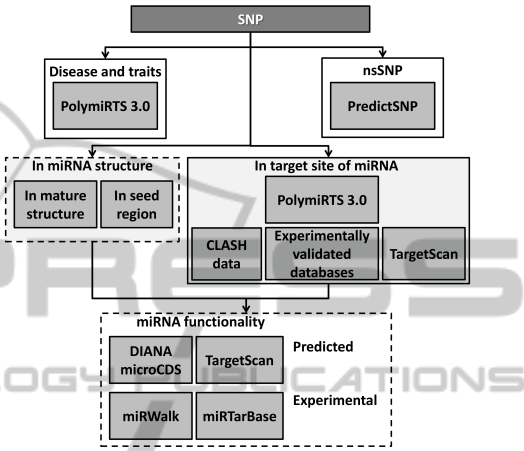

Figure 2: Scheme of data flow in SNP-miRNA interaction identification.

3 RESULTS AND DISCUSSION

Quality control revealed that one of the SNPs was missing in 91% cases and it was removed from further analysis. None outliers were detected in qPCRs. The total of 472,712 SNPs was considered during the BBC3 gene expression interaction modelling due to the lack of diveristy in the analysed samples for 94,383 SNPs. Table 1 includes the number of SNPs representing three types of modelled interactions between BBC3 gene expression level and genotyped polymorphisms (genotype, dominant and recessive). For both endpoints (0Gy and FCH) number of SNPs with particular interaction model being minimum p-value was given in the row named Best model chosen, while the next row gives the number of SNPs significant at α=0.05, split by SNP-BBC3 gene interaction model type.

While analysing the expression of BBC3 gene, the majority of SNPs represent dominant or recessive model of interaction with FDR level around 50%. All the polymorphisms with minimal p-value being less than 0.05 were considered as the candidate SNPs related to the radiosensitivity phenomena. After applying the inclusion criteria defined for the final two sets

Table 1: The results of the model selection for BBC3 at both endpoints - 0Gy and FCH.

	G	D	R	TOTAL
Total	183,965	309,155	348,188	841,308
		0Gy		
p<0.05	9,614	37,672	42,559	89,845
FDR [%]	95.68	41.03	40.91	46.81
		FCH		
p<0.05	8,833	31,084	34,440	74,357
FDR [%]	100	49.73	50.55	56.57
		0Gy		
Best model	2,462	215,268	254,930	472,712
p<0.05	1,550	35,073	39,865	76,488
		FCH		
Best model	2,670	215,590	254,452	472,712
p<0.05	1,525	29,034	32,355	62,914

G - Genotype model; D - Dominant model; R - Recessive model.

of candidate radiosensitivity related SNPs, SET 1 included 40,953 SNPs, while SET 2 consists of 21,961 SNPs.

3.1 Functional Analysis

3.1.1 SNP-gene Interactions

Table 2 presents detailed information about functionality of significant SNPs at two investigated endpoints.

Table 2: Transcriptomic location of candidate SNPs SET 1 and SET 2.

No. of SNPs in particular transcriptomic location					
		SET 1	[%]	SET 2	[%]
TOTAL		40,953	100	21,961	100
Total functional		17,096	41.8	9,169	41.8
intron		15,413	37.6	8,271	37.7
exon	synSNP	105	0.3	69	0.3
	nsSNP	244	0.6	121	0.6
UTR3'		418	1.0	223	1.0
UTR5'		67	0.2	29	0.1
nearGene3' *		83	0.2	34	0.2
nearGene5' **		287	0.7	171	0.8
splice3'		1	0.01	0	0.0
splice5'		5	0.01	1	0.01
frameshift		1	0.01	0	0.0
STOP codon loss		1	0.01	0	0.0
ncRNA		94	0.2	53	0.2
cds-reference		377	0.9	197	0.9

* nearGene3' - within 3' 0.5kb to a gene;

** nearGene5' - within 5' 2kb to a gene.

Initially, the impact of nonsynonymousSNP (nsSNP) was investigated by PredictSNP software and the results are presented in table 3. For all deleterious SNPs literature study was performed and for SET 1 three of them occur in genes highly relevant and affiliated to cancer processes: AMACR (Jianq et al., 2013), SERPINB5 (Kapoor, 2014), ABCC11 (Yamada et al., 2013): In SET 2 only one gene is highly relevant to cancer processes: TLR6 (Miedema et al., 2012).

Table 3: The percentage of deleterious nsSNPs in SET 1 and SET 2 candidate SNPs.

nsSNP predictions		
	SET 1	SET 2
Total	244	122
Deleterious	27	20
[%]	11.07	16.39

Additionally, both groups of genes with significant functional SNP-gene interactions, equal to 5,450 in case of SET 1 and 3,505 for SET 2, were investigated on the overrepresentation of KEGG pathways and the summary of that analysis presents table 4. In general, 45 KEGG pathways are overrepresented either in genes related to SET 1 or SET 2. Twenty two of them (48.89%) are significantly overrepresented in both gene sets. Among 32 significantly overrepresented KEGG pathways for genes disregulated by candidate functional SNPs from SET 2, the highest odds ratio was noticed for ko00604 (Glycosphingolipid biosynthesis, OR=4.51, p-value=0.010638) and ko00532 (Glycosaminoglycan biosynthesis, OR=3.68, p-value=0.012608) which is consistent with the latest literature based reports on the strong relation between these two processes and irradiation or cancerogenesis (Aureli et al., 2014; Hirshoren et al., 2014). One of the most significant overrepresented pathways was ko04360 (Axon guidance, OR=2.97, p-value=3.32E-07), process strongly related to local invasion and metastatic spread of the tumour (Ochi et al., 2002). All these three pathways were also significantly overrepresented among genes modified by SNPs from SET 1 (p-values equal to 0.027915, 0.003554, and 2.75E-07 for ko00604, ko00532, and ko04360 respectively). While looking at the signal pathways differentially represented in both gene sets, ko04810 (Regulation of actin cytoskeleton), ko04520 (Adherens junction), and ko05200 (Pathways in cancer) are overrepresented in SET 1 - related to the response to irradiation with no relation to 0Gy, and are not enriched in SET 2, which includes genes related to both to background (0Gy response) and response to irradiation level (FCH) (Bansal et al., 2014). The opposite analysis - looking for pathways overrepresented in SET 2 and not enriched in SET 1 brings ko04540 (Gap junction) and ko04210 (Apoptosis) known as re-

lated to radioadaptive response (Nenoi et al., 2014).

The detailed analysis of pathways overrepresented in both SET 1 and SET 2, three signal pathways, highly relevant to cancerogenesis, are overrepresentaed in both groups of genes - SET 1 and SET 2. Calcium signalling pathway (ko04020) plays the main role in cell signalling and is thought, for example, to have an impact for preventing metastases in breast cancer (Davis et al., 2014). MAPK signalling (ko04010) is the main path responsible for cell communication and reaction for stress. Many types of cancers have mutations in genes in MAPK pathway (Dhillon et al., 2007). The third one, focal adhesion (ko04510) has recently been identified as key determinat of cancer cell resistance to radio- and chemotherapy (Eke and Cordes, 2014).

Table 4: Details of KEGG pathways overrepresentation analysis among genes modified by SET 1 and SET 2 candidate nsSNPs.

KEGG pathways		
	SET 1	SET 2
No. of SNPs	17,096	21,961
No. of genes	5,450	3,505
No. of analysed KEGG pathways	232	227
No. of overrepresented KEGG pathways	35	32
FDR [%]	33.14	35.47

3.2 SNP-miRNA-gene Interactions

Summary of the results obtained presents table 5. Novel comprehensive approach allows to identify both types of interactions (polymorphism inside mature miRNAs and in target sites) which cannot be obtained with the use of standard data analysis systems, and which significantly increases the number of observed interactions.

Table 5: Summary of the comprehensive SNP-miRNA interaction analysis for two SNP sets SET 1 and SET 2.

No. of miRNA and polymorphisms interactions		
	SET 1	SET 2
SNPs in miRNA structure		
mature miRNA	13	2
seed region	4	2
SNPs in target site of miRNA		
CLASH	9	8
experimentally validated	2	2
in silico predicted	414	218

Several involved in cancer progression miRNAs, with polymorphisms in mature structure were found. The most relevant are: hsa-let-7a-3p with rs12326928 (p-value=0.0101; strong impact on breast cancer) (Yu et al., 2007)), hsa-miR-519c-5p with rs1816087 (p-value=0.0101; regulate human breast cancer resistance protein) (Li et al., 2011)), hsa-miR-512-3p with rs4145874 (p-value=0.0119; regulation of genes associated with cancer) (Chen et al., 2010)), and hsa-miR-22-5p with rs9828426 (p-value=0.0482; regulation of genes associated with breast cancer (Patel et al., 2011)).

Exemplary polymorphism modifying mature miRNA among those found for both SET 1 and SET 2 candidate SNP sets is rs2974617 (p-value=0.0152). It modifies the mature structure of hsa-miR-4796-5p and is located in TRIM36 gene. This gene and its products are members of tripartite motif (TRIM) family. Most of the genes from TRIM family are observed being differentially expressed in many types of cancers. The TRIM36 has a significant role in chromosome segregation and cell cycle regulation (Hatakeyamai, 2011). hsa-miR-4796-5p is not well described in literature. However, we found 40 potential target genes. This group was investigated by overrepresentation analysis of GO and KEGG pathways. The most important gene ontology term is GO:0097237 (cellular response to toxic substance; p-value=0.038) and already mentioned ko04020 (calcium signalling pathway; p-value=0.015).

The parallel analysis was performed to find significant polymorphisms located in miRNAs target sites. While looking at the results obtained with the use of CLASH database, SET 1 contains, for example, polymorphism rs11556080 (p-value=0.0270), which changes the regulation of CD99 gene. CD99 gene is located on X chromosome and unlikely to most of genes, it does not undergo X inactivation. CD99 is found in tumour cell of Ewing's sarcoma. Its knockdown reduces the tumour progression (Rocchi et al., 2010). The recent studies suggest CD99 as a biological marker for non-small lung cancer (Edlund et al., 2012). This dysregulation can be connected with the distribution of target site for hsa-miR-877-3p. The results of DIANA software (mirPath, 0.8 MicroT threshold) suggest that it can interact with genes connected with cancer (ko05200 pathways in cancer; p-value=0.006) and ko04115 (p53 signaling pathway; p-value=0.017) - figure 3. The SET 2 contains, among the others, rs989902 and rs184967 located respectively in PTPN13 and MSH3 genes. The first polymorphism can disturb target site for hsa-miR-186-5p (role in human colon carcinoma cells (Chen et al., 2013)), the second hsa-miR-92b-3p (connected with brain cancer and metastasis (Nass et al., 2009)). Both genes are highly

relevant for investigated trait. PTPN13 plays the role in the process of metastasis in lung cancer (Han et al., 2013), where MSH3 is one of the main genes responsible for miss-match in the repair process. Also, gene MSH3 was found as a candidate to describe the radiosensitivity phenomena (Mangoni et al., 2011). Using PolymiRTS Database 3.0 we obtained the information on relations of significant polymorphisms to different diseases (presented in table 6).

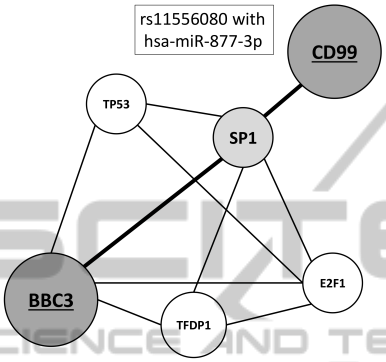

Figure 3: Pathway interaction between BBC3 and dysregulated gene CD99 based on genemania.org.

Table 6: Number of diseases and traits associated with polymorphisms from SET 1 and SET 2.

	SET 1	SET 2
No. of diseases/traits interactions	283	127
No. of unique diseases/traits	144	77
No. of cancer disease interaction	21	5

The comparison between novel comprehensive approach and SNPinfo based one reveals, that applying the detailed analysis of SNP-miRNA interactions combined with the integrative PredictSNP algorithm significantly increases the number of candidate functionally validated SNPs by 222 SNPs for SET 1 (92.88%, from 239 to 461) and by 150 SNPs for SET 2 (96.00%, from 125 to 245) - table 7.

Table 7: Summary of novel functional analysis for SET 1 and SET 2.

	SET 1	SET 2
TOTAL	461	245
Deleterious nsSNPs	27	20
SNPs in target site	421	223
SNPs in miRNA	13	2

4 CONCLUSIONS

We developed the novel comprehensive technique improving significantly the results of the analysis on genotype-phenotype interactions. Direct links between genotype results and gene expression levels were enriched by detailed analysis of SNP-miRNA-gene interactions at both mature miRNA structure/seed region and target binding site level. The presented analysis can filter out non-functional SNPs from extremely large number of relevant polymorphisms resulting of GWAS analysis.

The proposed technique was applied to the problem of searching for genetic signature of radiosensitivity. Eight polymorphisms highly relevant to the process of description of the radiosensitivity phenomena were obtained, majority of them were indirectly validated during the literature study

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DECLARATION OF INTEREST

The authors alone are responsible for the content and writing of the paper.

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