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ORIGINAL PAPER

Meta-analysis and prioritization of human skin pigmentation-associated GWAS-SNPs using ENCODE data-based web-tools

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Abstract

Skin pigmentation in human is a complex trait, which varies widely, both within and between human populations. The exact players governing the trait of skin pigmentation remain elusive till date. Various Genome Wide Association Studies (GWAS) have shown the association of different genomic variants with normal human skin pigmentation, often indicating genes with no direct implications in melanin biosynthesis or distribution. Little has been explained in terms of the functionality of the associated Single-Nucleotide Polymorphisms (SNPs) with respect to modulating the skin pigmentation phenotype. In the present study, which, to our knowledge, is the first of its kind, we tried to analyze and prioritize 519 non-coding SNPs and 24 3′UTR SNPs emerging from 14 different human skin pigmentation-related GWAS, primarily using several ENCODE-based web-tools like rSNPBase, RegulomeDB, HaploReg, etc., most of which incorporate experimentally validated evidences in their predictions. Using this comprehensive, in-silico, analytical approach, we successfully prioritized all the pigmentation-associated GWAS-SNPs and tried to annotate pigmentation-related functionality to them, which would pave the way for deeper understanding of the molecular basis of human skin pigmentation variations.

Keywords Pigmentation · GWAS · ENCODE · SNP · Melanin

Introduction

Skin pigmentation in human is one of the most complex quantitative traits ever studied. Epidemiological studies had shown that skin pigmentation is a polygenic trait with high heritability [6, 13, 18]. It shows broad range of variations both within and between populations. Till date, various Genome Wide Association Studies (GWAS) were conducted to elucidate the underlying players and the molecular bases of the skin pigmentation variations within and between populations. These efforts although could implicate various hitherto known and unknown players in the melanin biosynthesis and distribution, little evidence could be gathered with respect to the functional attributes of the associated SNPs [3, 7, 11, 15, 19, 20,

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28, 32, 35, 39–41, 45]. As in case of other traits and diseases, the pigmentation-related GWAS results have often come up with intronic and intergenic variants, too, distally located from the known genes or transcribed regions. Thus, assigning proper functional or regulatory roles has always been a confounding job to undertake in case of such associated variants. With the release of ENCODE project data (freely available at https://www.encodeproject.org) [9, 10, 35, 41], it has become easier to predict the array of functional contributions of genomic variants in terms of their regulatory roles. ENCODE data incorporates 5C, ChIA-PET, Hi-C, DNase-seq, FAIRE-seq, ATAC-seq, ChIP-seq, RNA-seq, CLIP-seq, and RIP-seq data from 10,054 human biosample types, which give its users an access to functionally validate experimental data entailing different regulatory aspects of genomic variants like DNA binding, DNA accessibility, RNA binding, DNA methylation, 3D chromatin structure, transcriptional alterations, etc. It is worth mentioning here that there are freely available tools in the Internet, which incorporate the predictions of nucleotide variants based on ENCODE data. rSNPBase (http://rsnp.psych.ac.cn) [17] is such a database that "provides reliable, comprehensive, and user-friendly regulatory annotations on rSNPs" (regulatory Single-Nucleotide Polymorphisms). It assists researchers "in selecting candidate SNPs for further genetic studies (especially for QTL studies), identifying the causal variants of certain phenotypes, and exploring in-depth molecular mechanisms." All SNPs searched through it are annotated with reference to experimentally (ENCODE data) supported regulatory elements, incorporating a wide range of regulation types. On the other hand, RegulomeDB (http://www.regulomedb.org) [1] involves manually curated regions that have been experimentally characterized to be involved in regulation, ChIP-seq information for a variety of important regulatory factors across diverse set of cell types, chromatin state information across different cell types, and eQTL information to allow the association of distal sites with gene promoters. Another freely available web-based tool, HaploReg v4.1 (http://archive.broadinstitute.org/mammals/haploreg/haploreg.php) [43, 44] helps to search SNPs within LD blocks based on the chromatin state data along with conservation and regulatory motif alteration data for all query SNPs. With our present study, for the very first time, we tried to analyze and prioritize all the human skin pigmentation-associated GWAS-SNPs based on ENCODE-based in-silico web-tools which incorporate experimentally validated evidences in their prediction algorithm. Through our analyses, we have successfully been able to prioritize SNPs with higher chances of being functionally associated with normal human skin pigmentation regulation pathways. We also tried to annotate pigmentation-related functions for the genes and SNPs associated.

Materials and methods

Selection of SNPs

We searched GWAS catalog (https://www.ebi.ac.uk/gwas) [26] and PubMed for the available literature on human skin pigmentation and found 14 different studies, conducted worldwide since 2007. The search string for PubMed search has been "HUMAN SKIN PIGMENTATION AND GWAS". After searching of literature was done, a table was made to include all the significant SNPs from 14 different GWA studies. Repeating of the same SNP IDs from different GWAS was carefully checked. Curated SNP IDs from both the searches were pooled together and submitted as Supplementary material 1.

Analysis in rSNPBase

It is already mentioned that rSNPBase helps to identify potential target loci based on spatio-temporal and experimental eQTL (expression Quantitative Trait Loci) labels. Linkage disequilibrium (LD) correlations between SNPs are also analyzed to get the results of probable functional regulatory effects of query SNPs in the form of a SNP-set basis, rather than individual single result for each query SNP [17]. In this study, we used "List search" option of rSNPBase (http://rsnp.psych.ac.cn/listSearch.do). SNP IDs were submitted in the search box in enter delimited format, followed by the searching step. It is to be noted that, for each query, the "rSNP" column of the result page gave "yes" outcome if any experimental data are available; otherwise, the outcome in the "rSNP" column reads as "no". We did not include outputs with "no" in our filtration.

Analysis in regulomeDB

SNPs that were found to have regulatory effects during rSNPBase analysis were searched further in RegulomeDB. RegulomeDB provides a heuristic scoring system, with increasing confidence, for each query variant based on its functional location and consequence, if any. The scoring system ranges from 1 to 6. Score category 1 and all its sub-categories indicate that the variant is "likely to affect binding and linked to expression of a gene target". Score category 2 along with its sub-categories indicates that the query variant is "likely to affect binding". Categories 3–6 represent those variants for whom lesser evidences are there with respect to their regulatory potentials. The "dbSNP IDs" option of RegulomeDB

was used in this study and all the SNP IDs were submitted in enter delimited format.

Analysis in HaploReg v4.1

SNP Ids that obtained score 1 and score 2 during RegulomeDB analysis were pooled together and further searched in HaploReg v4.1. For searching purpose, we created and uploaded a text file with SNP IDs in enter delimited format having only one SNP ID per line. Before searching, the LD threshold value of HaploReg was set to 0.6 and the other setting options were left unchanged. HaploReg v4.1 was used only to check if any of our prioritized SNP was in LD with the other query SNP or not. Therefore, it is worth mentioning that HaploReg v4.1 was not used to prioritize SNPs.

Searching targets of regulations and cell line-based evidences

After prioritization of GWAS-SNPs using the aforementioned tools, we tried to find the target loci for each of the prioritized SNPs. We followed the hyperlinks provided by rSNPBase (on the SNP identifiers) and checked the "SNP Report" page. Types of regulations and targets of such regulations were checked for each query rSNP from "SNP Report" page. We also searched for cell line-based or tissue-specific evidences, if any, for all the different types of regulatory predictions from the hyperlink provided with RegulomeDB score for each of the rSNP (with RegulomeDB score 1 or 2). The hyperlink led us to SNP data sheets for each of the query SNP and provided us links for gene expression data of GTEx RNA-seq (covering 53 tissues, 8555 samples from 570 donors) from where we could check gene expression data of our gene of interest containing our query SNP [4, 16, 31].

STRING v10.5 analysis

STRING (https://string-db.org) assesses the functional interactions between proteins based on systematic co-expression analyses, detection of shared selective signals across genomes, automated text-mining of scientific literature, and computational transfer of interaction knowledge between organisms based on gene orthology [38]. With STRING, we tried to assess how many of the prioritized SNP harbouring loci and their target loci actually interacted with already known pigmentation-related loci. Thus, we tried to create a list of loci that were already known for their roles in human skin pigmentation. We searched KEGG PATHWAY Database (http://www.genome.jp/kegg/pathway.html) for known human skin pigmentation-related loci and included loci names from tyrosine metabolism (KEGG identifier: hsa00350) and melanogenesis (KEGG identifier: hsa04916) pathways. We also found a database, "Harmonizome", which was found to incorporate "integrated knowledge about genes & proteins" and enlisted gene/protein names for different human traits and human diseases [34]. In Harmonizome, we found a list of gene names as "Abnormality Of Skin Pigmentation Gene Set" (http://amp.pharm.mssm.edu/Harmonizome/gene_set/abnormality+of+skin+pigmentation/GWASdb+SNP-Phenotype+Associations). We made a pool of different loci names from all the three sources mentioned and submitted them in STRING search box along with the loci names of prioritized SNPs.

Results

Final results from both the searches (GWAS catalog and PubMed) yielded 543 different SNPs of which 519 were found to be non-coding SNPs and 24 SNPs from 3′ UTR. All the SNPs obtained from the 14 GWAS were assessed through a pipeline, as depicted in Fig. 1. Below, we discussed the results of individual web-based prediction tools which were used for analysis and prioritization of the GWAS-SNPs selected.

Out of 519 non-coding SNPs and 24 SNPs from 3′UTR, 312 non-coding SNPs and 22 SNPs of 3′UTR were predicted to be Regulatory Single-Nucleotide Polymorphisms (rSNPs) by rSNPBase. These 312 non-coding SNPs and 22 SNPs of 3′UTR were next submitted in RegulomeDB search box, individually, to find their extent of regulatory potential. Eventually, 23 non-coding SNPs and 2 (two) 3′UTR SNPs were identified within score categories 1 and 2. These 25 variants belonged to 12 different loci.

Next, we checked the target loci for all the 25 prioritized SNPs from the hyperlinks provided with rSNPBase results. Altogether, 25 prioritized SNPs were found to target 58 different loci through 106 modes of regulations (Supplementary material 2). Our results also showed that, for 22 prioritized SNPs (out of the 25 SNPs), the nearest gene or the gene to which the SNP belongs was the target gene; and only 1 SNP (rs16963682) was found to target a different gene, exclusively (see Supplementary material 2).

The 25 SNPs (which were selected after RegulomeDB analyses) were searched, in HaploReg v4.1, for their potential of being in LD with each other. Out of the 25 query SNPs, 8 were found to be in LD with the other query SNPs. However, we did not exclude any SNPs that were in LD with the other query SNPs for our next set of analyses (see Supplementary material 2).

Our cell line-based evidence searching results indicated that out of 25 prioritized SNPs targeting 58 different loci through 106 modes of regulations, 9 SNPs (from 5 underlying genes) targeting 8 loci (6 protein coding: *CEP152, ATP8B4, MFSD12, TUBB3, MC1R*, and *TYRP1* and 2

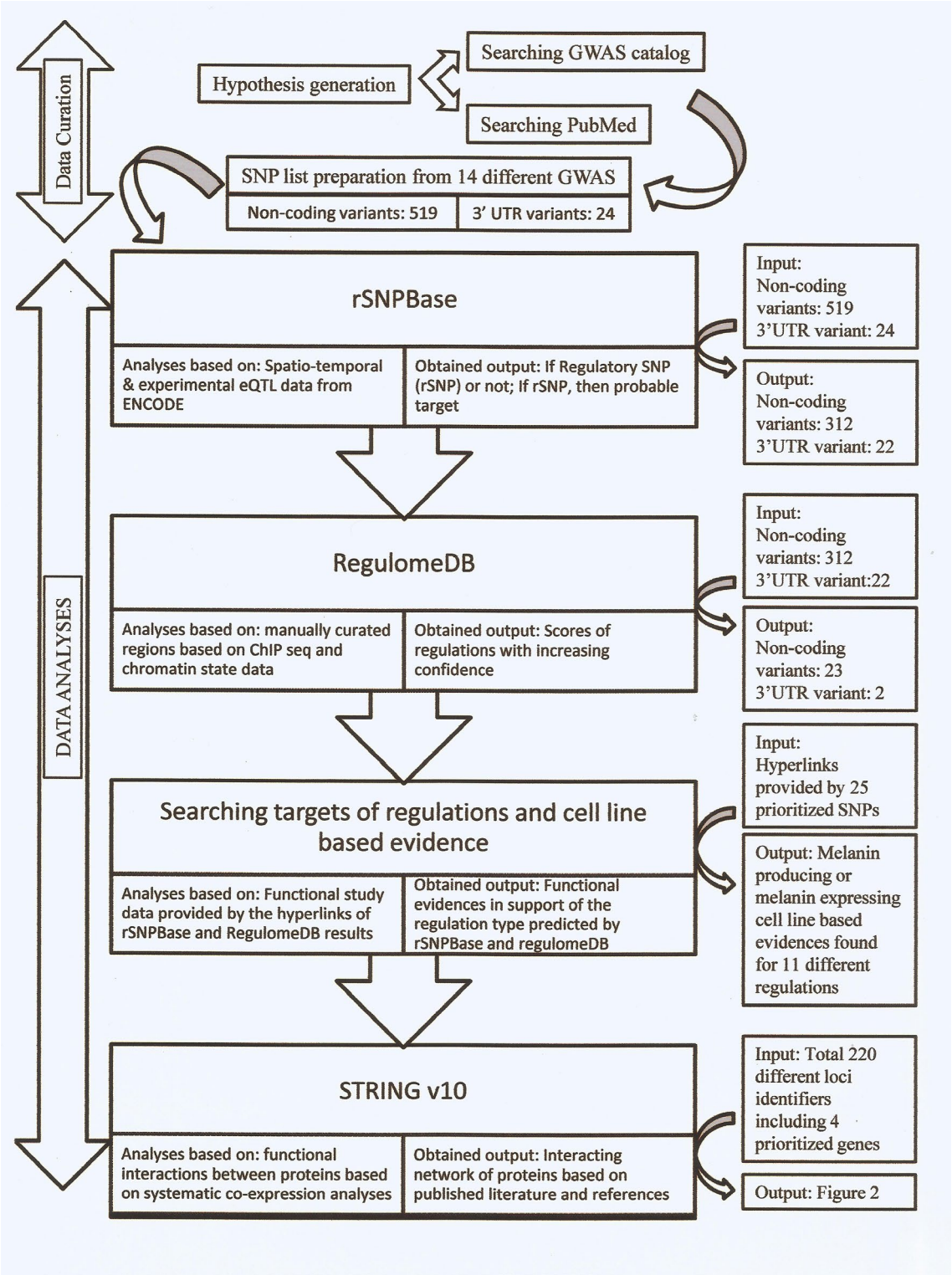


Fig. 1 Scheme of analyses used for prioritization of skin pigmentation-associated GWAS-SNPs (In the figure only, those ENCODE-based tools are mentioned which were used for prioritization process)

pseudo-gene coding) were found to be validated in melanin producing and/or expressing cell line(s) (Supplementary material 2).

Finally, we wanted to check if these six protein coding genes had any functional association in between them or with the known melanin biosynthetic and distribution pathway. We submitted all the six protein identifier codes (*CEP152, ATP8B4, MFSD12, TUBB3, MC1R*, and *TYRP1*) which were found to be targets of the 9 SNPs prioritized so far, for STRING analyses along with 216 different protein identifier codes which were pooled using KEGG and Harmonizome (already mentioned in "Materials and methods" section). It is worth mentioning here that TYRP1 and MC1R were well characterized with respect to their functions in human skin pigmentation, and they were also included in the 216 gene set. Out of the six protein targets, four, viz., TUBB3, CEP152, TYRP1, and MC1R, were found to have connectivity with already known pigmentation pathway proteins (Fig. 2).

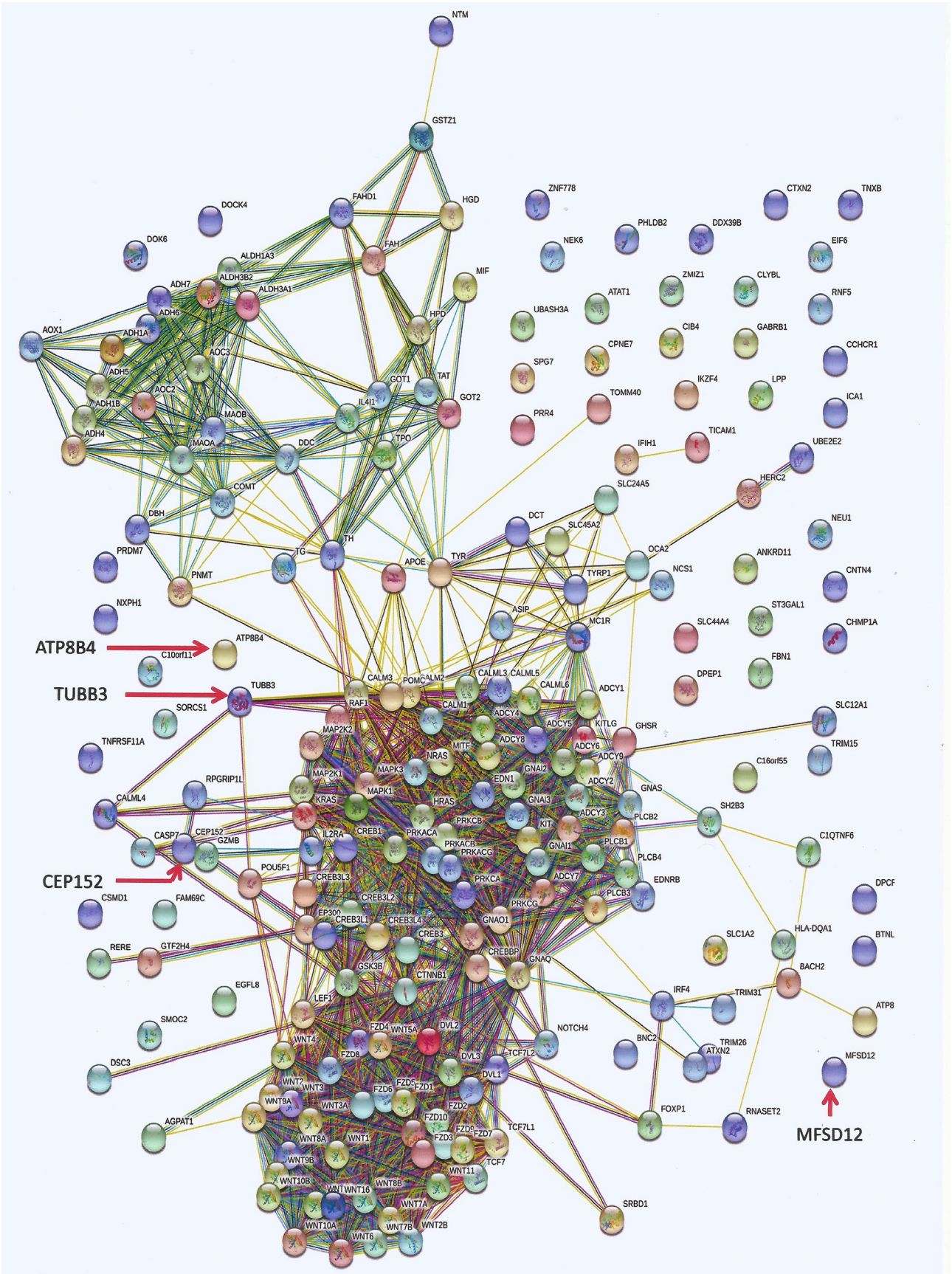
Table 1 gives us a brief overview of the prioritized SNPs, their targets, and modes of regulating those targets.

Discussion

Through an in-silico pipeline, here, we have tried to assess the functional relevance of SNPs, reported in the GWAS studies, to be associated with human skin pigmentation, in terms of their regulatory potential. Out of 543 SNPs identified from the GWAS data sets, we could predict 25 SNPs (of which 8 SNPs were found to be in LD other predicted SNPs) targeting 58 different loci through 106 modes of regulations, of which 9 SNPs targeting 8 loci (6 protein coding and 2 pseudo-gene coding) were found to be validated in melanin producing and/or expressing cell line(s). Out of these 8 loci, 6 had annotated genes (*TYRP1, MC1R, TUBB3, CEP152, MFSD12*, and *ATP8B4*) of which 2 were already known to have roles in human pigmentation, i.e., *TYRP1* and *MC1R*. TYRP1 catalyzes the oxidation of 5, 6-dihydroxyindole-2-carboxylic acid (DHICA) into indole-5,6-quinone-2-carboxylic acid in the presence of bound Cu^{2+} ions [24]. TYRP1 may also regulate or influence the type of melanin synthesized [22, 33]. Again, it is known that alpha-Melanocyte-stimulating hormone (alpha-MSH) regulates skin and hair pigmentation by modulating the activity of MSH receptor (MC1R) [12]. When the six protein identifiers (protein coding target genes of the prioritized SNPs) were run in STRING v10.5 along with 216 known protein identifiers associated with melanogenesis and tyrosine metabolism taken from KEGG pathway, apart from TYRP1 and MC1R, TUBB3 and CEP152 were also found to have some connections with the known players of melanogenesis/tyrosine metabolism. TUBB3 was found to interact with CALML5 [ENSP00000369689] and CALML6 [ENSP00000304643]. Calmodulin like skin proteins are already known as markers for keratinocyte differentiation [30]. Interestingly, TUBB3 resides in a very close proximity to the 3′ UTR region of MC1R and intergenic splicing between MC1R and TUBB3 has been reported [8, 21]. Interestingly, treatment with the MC1R agonist α-MSH or activation of the stress response kinase p38-MAPK, both key players associated with ultraviolet radiation dermal insult and subsequent skin tanning, resulted in a shift in expression from MC1R in favour of chimeric MC1R-TUBB3 isoforms in cultured melanocytes. It has been, thereby, proposed that these chimeric proteins serve to equip melanocytes with novel cellular phenotypes required as part of the pigmentation response [8, 21]. On the other hand, CEP152 or centrosomal protein of 152 kDa plays very important role in cell division. It is necessary for centrosome duplication involving CEP63, CDK5RAP2, and WDR62 through a stepwise-assembled complex at the centrosome that recruits CDK2 required for centriole duplication [23]. After STRING analyses, CEP152 was found to interact with PRKACA [ENSP00000309591] and RPGRIP1L [ENSP00000369257]. PRKACA or cAMP-dependent protein kinase catalytic subunit alpha is involved in phosphorylation of a large number of substrates in the cytoplasm and the nucleus (Source: http://www.uniprot.org/uniprot/P17612). PRKACA has been found to interact with CALM3 [ENSP00000291295] and ADCY1 [ENSP00000297323], both of which are involved with maintenance of intra-cellular Ca^{2+} level, which is known to regulate melanin production and transport (not only transport) in vitro [2, 29, 37].

In this respect, the role of MFSD12 is worth mentioning, although we did not find it within the STRING interactome, because, as mentioned earlier, STRING analysis assesses the functional interactions between proteins based on systematic co-expression analyses, detection of shared selective signals across genomes, automated text-mining of scientific literature, and computational transfer of interaction knowledge between organisms based on gene orthology. MSFD12 is a lysosomal transmembrane solute transporter protein [27] and has been recently found to suppress eumelanin biogenesis in melanocytes from lysosomes in mice cell line [7].

ATP8B4 is a component of a P4-ATPase flippase complex which catalyzes the hydrolysis of ATP coupled to the transport of aminophospholipids from the outer to the inner leaflet of various membranes and ensures the maintenance of asymmetric distribution of phospholipids (UniProt http://www.uniprot.org/uniprot/Q8TF62). It is worth mentioning here that rare variants of ATP8B4 had been found as risk factors for development of Systemic Sclerosis (SSc) [14] and hyperpigmentation is a one of the common pathogenic manifestations of SSc [5]. Thus, it seems that, directly or


ATP8B4
TUBB3
CEP152
MFSD12
NTM
GSTZ1
FAHD1
HGD
DOCK4
DOK6
FAH
ALDH1A3
ALDH3B2
ALDH3A1
ADH7
ADH6
ADH1A
AOX1
AOC3
ADH5
AOC2
ADH1B
ADH4
MAOB
MAOA
MIF
HPD
GOT1
TAT
GOT2
IL4I1
DDC
TPO
COMT
DBH
PRDM7
PNMT
NXPH1
TG
TH
APOE
TYR
DCT
SLC45A2
TYRP1
OCA2
NCS1
ASIP
MC1R
C10orf11
ATP8B4
TUBB3
SORCS1
TNFRSF11A
RPGRIP1L
CALML4
CASP7
CEP152
GZMB
POU5F1
CSMD1
FAM69C
RERE
GTF2H4
EGFL8
SMOC2
DSC3
AGPAT1
CALM3
POMC
CALM2
CALML3
CALML5
CALML6
ADCY1
RAF1
CALM1
ADCY4
ADCY5
KITLG
GHSR
MAP2K2
MITF
ADCY8
ADCY6
ADCY9
NRAS
MAP2K1
MAPK3
GNAI2
EDN1
ADCY2
GNAS
KRAS
MAPK1
HRAS
GNAI3
ADCY3
PLCB2
PRKCB
KIT
IL2RA
CREB1
PRKACA
PRKACB
PRKACG
GNAI1
PLCB1
PLCB4
CREB3L3
PRKCA
ADCY7
EDNRB
PLCB3
CREB3L2
EP300
CREB3L1
CREB3L4
PRKCG
GNAO1
CREB3
CREBBP
GNAQ
GSK3B
CTNNB1
LEF1
FZD4
WNT5A
DVL2
NOTCH4
WNT4
FZD8
DVL3
TCF7L2
WNT2
WNT3
FZD6
FZD5
FZD1
DVL1
WNT9A
WNT3A
FZD2
FZD10
FZD9
FZD7
TCF7L1
WNT8A
WNT1
FZD3
WNT9B
TCF7
WNT11
WNT10B
WNT16
WNT8B
WNT7A
WNT10A
WNT7B
WNT2B
WNT6
SRBD1
ZNF778
CTXN2
TNXB
PHLDB2
DDX39B
NEK6
EIF6
ZMIZ1
CLYBL
ATAT1
RNF5
UBASH3A
CIB4
GABRB1
CPNE7
CCHCR1
SPG7
IKZF4
LPP
TOMM40
ICA1
PRR4
TICAM1
IFIH1
UBE2E2
HERC2
SLC24A5
NEU1
ANKRD11
CNTN4
ST3GAL1
SLC44A4
CHMP1A
FBN1
DPEP1
SLC12A1
TRIM15
C16orf55
SH2B3
C1QTNF6
DPCF
SLC1A2
HLA-DQA1
BTNL
BACH2
IRF4
TRIM31
ATP8
BNC2
TRIM26
ATXN2
FOXP1
RNASET2

◂**Fig. 2** STRING v10.5 network (four target proteins, i.e., TUBB3, CEP152, MFSD12, ATP8B4, of prioritized SNPs are marked with red arrow. While TYRP1 and MC1R are not marked, but they could be seen within the interactome, interacting with the other proteins)

indirectly, ATP8B4 is involved with human pigmentation alteration.

Herein, we should re-emphasize on the fact that our study wished to check the regulatory potential of non-coding SNPs taken from GWAS of human skin pigmentation. Thus, a few genes like tyrosinase and SLC24A5 which have been known to contribute in population-level pigmentation variation, mainly through their coding SNPs, did not come up in our prioritized gene set.

Outcomes of the study

Globally, different GWA studies have been trying to unearth unknown genomic variants regulating normal human skin pigmentation, but, in many cases, they lack functional insights. Our study has tried to provide a deeper insight into the currently available GWAS variant repertoire. Using freely available and ENCODE-based web-tools (all of which incorporate data based on different functional experiments carried on a genomic scale), we have tried to prioritize and check the functional attributes (regulatory roles) of all the skin pigmentation-related GWAS-SNPs and the genes which they regulate. The success of our prioritization process is reflected in finding TYRP1 and MC1R as two of the prioritized loci, which have well-established role to regulate human skin pigmentation. On the other hand, finding TUBB3 and CEP152 with our prioritization pathway suggests that, apart from direct involvement in melanin biosynthesis, processes like differentiation of keratinocyte and regulation of intra-cellular Ca^{2+} level might also determine the final outcome of human skin pigmentation. Finally, we need to emphasize on the fact that though we could not find ATP8B4 and MFSD12 within the STRING-based 'melanogenic' interactome, available literatures support their roles in pigmentation alteration. We opine that the method implied in this study for prioritization of human skin pigmentation-associated GWAS-SNPs would be fruitful for prioritization of the other complex phenotype-associated SNPs.

Table 1 List of GWAS-SNPs, their target genes, and modes of regulations as prioritized through the proposed *in-silico* pipeline

rSNP ID	Gene/nearest gene	Genomic location	Target of regulation	Potential mode of regulation
rs7182710	CEP152	5′ near gene	CEP152	Proximal transcriptional regulation
rs784416	CEP152	Intron	ENSG00000259216/AC084757.1/ Processed pseudo-gene	Proximal transcriptional regulation
rs7176696	CEP152	Intron	ENSG00000259670/AC012379.1/ Anti-sense RNA coding gene	Proximal transcriptional regulation
rs937171	ATP8B4	Intron	ATP8B4	Proximal transcriptional regulation
rs2762462	TYRP1	Intron	TYRP1	Proximal transcriptional regulation
rs10424065	MFSD12	Intron	MFSD12	Proximal transcriptional regulation
rs142317543	MFSD12	Intron	MFSD12	Proximal transcriptional regulation
rs3212369	MC1R	3′UTR	MC1R	Proximal transcriptional regulation
			ENSG00000258947/TUBB3	Proximal transcriptional regulation
rs3212368	MC1R	3′UTR	MC1R	Proximal transcriptional regulation
			ENSG00000258947/TUBB3	Proximal transcriptional regulation

3′UTR > 3 prime un-translated region; 5′ > 5 prime

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Compliance with ethical standards

Conflict of interest All the authors gave approval for submission of the current version of the manuscript for publication and have full access to the study data. Authors declare no conflict of interest with respect to this article.

Ethical approval and informed consent from patients Not applicable for this study.

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