DECODING THE GENETIC ALTERATIONS IN PRAME GENE FAMILY AND ITS ASSOCIATION WITH HEAD AND NECK SQUAMOUS CELL CARCINOMA

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ABSTRACT

Head and neck squamous cell carcinoma (HNSCC) is the most common form of cancer with an incidence rate greater in male than in female. Advancements in molecular diagnostics have identified several pathways which can have a direct or indirect role in the development and

progression of HNSCC. The PRAME (PReferentially Antigen expressed in MElanoma) gene family is yet another group of genes which has been recently implicated in HNSCC. The present study aims to identify the genetic alterations, the pattern of gene expression and the consequence of mutations in the *PRAME* family of genes in HNSCC patients. Several databases such as cBioportal, gnomAD, IMutant, PROVEAN were used to assess genetic alterations. The alterations included deep deletions, amplification, inframe, missense, truncating mutations. The gene showing the highest frequency of alteration (PRAME - 3%) was further assessed for its gene expression profile using the UALCAN database. The expression profile relative to normal samples was found to be significantly higher in HNSCC patients ($p = 1.11 \times 10^{-16}$). Further, the survival curve based on high and low/medium expression of the PRAME gene was assessed by Kaplan-Meier method. The analysis revealed a significant difference in the survival rate of patients with high and low/medicum level expression (0.0095). In addition, the high level expression was found to be associated with poor survival rate in HNSCC patients compared to those exhibiting low and medium level expression. In conclusion the study provides insights into the putative association of genes of the *PRAME* family with HNSCC. The preliminary results have to be further validated using experimental procedures.

Key words: head and neck cancer, association, PRAME gene expression, genetic alterations.

INTRODUCTION

Squamous cell carcinomas of the head and neck (HNSCCs) are an invasive genetically complex phenotype with an incidence rate reaching a steep increase in the developing nations. Despite several treatments options, the primary treatment choices for most patients are surgery and radiotherapy. But these therapies are associated with significant morbidity and a decline in

quality of life. Radiotherapy resistance is most commonly observed in HNSCC patients [1]. The human papillomavirus (HPV16,18) was unambiguously implicated in a subset of these malignant growths as a causative factor. Treatment options for an individual are decided based on some parameters such as the capability to tolerate treatment, concurrent sickness and the awaited practical results [2, 3]. The proportion of male and female ranges somewhere in the range of 2:1 and 4:1. Major malignant growths in the HNSCC are precipitated after the upper aerodigestive epithelium is exposed to carcinogenic agents. Such malignancies are unequivocally connected with certain hazard factors, *viz.,* smokeless tobacco, pan, gutka, alcoholism, and certain other environmental factors [4]. The identification of genetic alterations in crucial genes known to be associated with HNSCC would open new avenues towards identification of potential targets to develop diagnostic and therapeutic molecules. The PRAME (Preferentially Expressed Antigen in Melanoma) family of genes were found to be associated with solid tumors [5-7]. The protein was found to trigger cell mediated immune response in melanoma [8]. Despite the fact that PRAME is weakly expressed in normal tissues except for the testis, it is found to contribute to disease by modifying the retinol pathway [9]. A very recent study conducted by Szczepanski and colleagues demonstrated the association of PRAME gene expression with poor outcomes in HNSCC patients [10]. Another study has also documented the involvement of PRAME in the epithelial mesenchymal transition of triple negative breast cancer [11]. In this context, the present *in silico* study was designed to identify gene alterations and differential expression patterns in the *PRAME* gene family.

MATERIALS AND METHODS

Data source:

The present study follows a retrospective design of the observational study. The source of patient data was obtained from the database cBioportal [12,13]. The TCGA, Firehose legacy data set consisted of 528 cases of the head and neck squamous cell carcinoma, of which 512 tumor samples had sequencing and copy number alteration data. The demographic details of the patients have been given in Table 1. The database "HUGO Gene Nomenclature Committee at the European Bioinformatics Institute" (www.genenames.org/data/) contained a list of essential genes belonging to the *PRAME* gene family (23 genes). A user defined query of the list of genes in the PRAME gene family was submitted and the resulting oncoprint data was analysed further. Oncoprint data provided information on the type of alterations identified in the *PRAME* family of genes. The alterations included gene amplification, deep deletions and several forms of mutations and variations (Table 2; Figure 1).

gnomAD analysis:

Dataset gnomAD v2.1.1 consists of an array of 125,748 exomes and 15,708 individual sequencing genomes. Such research was used to search for the occurrence of the missense variants found in the HNSCC data in other persons for whom the sequencing data is available. Variations across 141,456 human exomes and genomes show the continuum of resistance and loss of function across human protein coding genes [14].

Protein stability and pathogenicity analysis:

The stability of the proteins upon substitution of one amino acid with the other was identified using I-Mutant suit 2.0 version. The predictions were based on the free energy change values (DDG). Any value below or less than 0 is considered to decrease the stability and values greater

than 0 is considered to increase stability [15]. The PROVEAN (Protein Variation Effect Analyzer) tool was used to predict the impact on the biological function of a protein upon substitution with an amino acid (Table 3) [16]. Any score below -2.5 is considered to pathogenic and a score above -2.5 is considered to be neutral.

UALCAN analysis:

The expression of the gene in HNSCC was analysed using the UALCAN (http://ualcan.path.uab.edu/cgi-bin/TCGA-survival1.pl?genenam) database (Figure 2 and 3). Gene expression data was expressed as transcripts per million (TPM) which is a normalization method for RNA- seq data. The TPM values used for the generation of box-whisker plots were also used to determine the significant difference between the groups. Survival effect analysis of gene expression were assessed using multivariate Kaplan- Meier survival analysis (Figure 4) [17].

RESULTS AND DISCUSSION

Oncoprint data demonstrated a similar pattern of gene amplification and deletion in *PRAME* and *PRAMENP* genes in thirteen HNSCC patients. Interestingly, four other patients also showed a similar pattern of gene alteration (Figure 1). Several mutations such as inframe, missense and truncating mutations have been identified. The gnomAD analysis revealed a few reported variants in *PRAMEF1 (rs149382773), PRAMEF4 (rs753793229), PRAMEF7 (rs779669158), PRAMEF10 (rs1167071023), PRAMEF12 (rs752095583, rs757917825)* and *PRAMEF18, (rs1384433084)* genes. The *PRAME* gene was found to harbour the highest frequency of alteration (3%). Several variants identified in the genes of the *PRAME* gene family was found to alter the protein stability resulting in pathogenic phenotypes. The present study is first of its kind

to report genetic variants and alterations in the PRAME family of genes in HNSCC patients (Table 2 and 3).

The gene expression profile of *PRAME* gene relative to normal sample was found to be increased significantly ($p = 1.11 \times 10^{-16}$). The differential expression pattern was also observed in different grades of tumor (Figure 3). This type of differential expression is indicative of the fact that the protein can be used as a marker for the diagnosis of progressive tumor. The survival of HNSCC patients based on the high and low/medium expression of *PRAME* gene also returned a significant p value of 0.0095, wherein overexpression of PRAME was associated with poor survival outcomes in HNSCC patients when compared to those exhibiting a low/medium gene expression. These results were in agreement with similar studies carried out in PRAME gene on multiple tumor types and populations. A study conducted by Yang and team investigated the impact of copy number variations on PRAME expression in multiple myeloma (MM) patients. Their results showed that 28% of patients showed over-expression of PRAME which also correlated with lower one year progression free survival when compared to patients with low expression levels. Therefore, they concluded by stating that overexpression of PRAME could act as an adverse prognostic factor in case of MM [18]. Another study designed to determine the frequency of expression of tumor associated antigens in non-small cell lung cancer (NSCLC) patients of Taiwan showed 59.2% of patients with squamous cell carcinoma (SCC) had expression of PRAME. Also the expression of PRAME was more frequent in SCC when compared to adenocarcinomas [19]. Xu et al, reported the expression of PRAME in salivary duct carcinoma (SDC) [20]. They observed a significant correlation of several immunological markers along with PRAME in tumor cells, thus proving the role of the gene in SDC. A very

recent study by Toyoma and colleagues proposed that high PRAME expression correlated with poor prognosis in mucosal melanomas [21].

Several molecular pathways have been implicated in PRAME mediated neoplastic transformation of the cells. It is a dominant repressor of signalling pathway involved in the retinoic acid metabolism. This process was found to inhibit differentiation of cells, arrest of proliferative capability and apoptosis. Overexpression in myeloid cells prevented its differentiation [22]. Recent reports have identified that PRAME could promote tumor initiation and progression through different molecular mechanisms. The involvement in transcriptional regulation of driver genes involved in tumor promotion is one important pathway which is worth mentioning [23]. Interactome studies on PRAME revealed that it recruits Cullin2 ubiquitin ligases which are involved in the process of transcriptional regulation, maintenance of telomere and modification of transfer RNAs viz., threonylcarbamoyladenosine (t6A) which decodes the Adenosine residues in mRNA [24-26]. Computational tools have been regarded as a boon to molecular biologist since an exhaustive collection of data is made available to the researchers to analyze. Numerous studies have been designed based on the preliminary results obtained from such simulations [27]. Despite the advantages listed, the study also suffers some limitations such as, (a) the patients recruited in the dataset is representative of most of the American population, hence the variants observed and their frequencies could differ among different populations world-wide, (b) the predictions about protein stability and pathogenicity may vary in an original biological system and hence more tools are required to arrive at a conclusion about the results obtained. With all the limitations addressed, the authors present with the preliminary data to provide evidence on the putative association of *PRAME* gene with HNSCC.

CONCLUSION

The *PRAME* is one of the most widely experimented cancer testis antigen in various cancers. The fact that the expression is restricted to somatic tissues, its expression in different types of cancer is worth investigating. The overexpression of PRAME is more often associated with the risk of metastasis and poor survival rate. Although considered to be an infamous protein molecule, PRAME has recently gained attention as a potential candidate for immunotherapy. Further investigations employing functional analysis of the variants and population wide screening would gather more information on the association of PRAME gene alterations with HNSCC.

Disclaimer regarding Consent and Ethical Approval:

As per university standard guideline, participant consent and ethical approval have been collected and preserved by the authors

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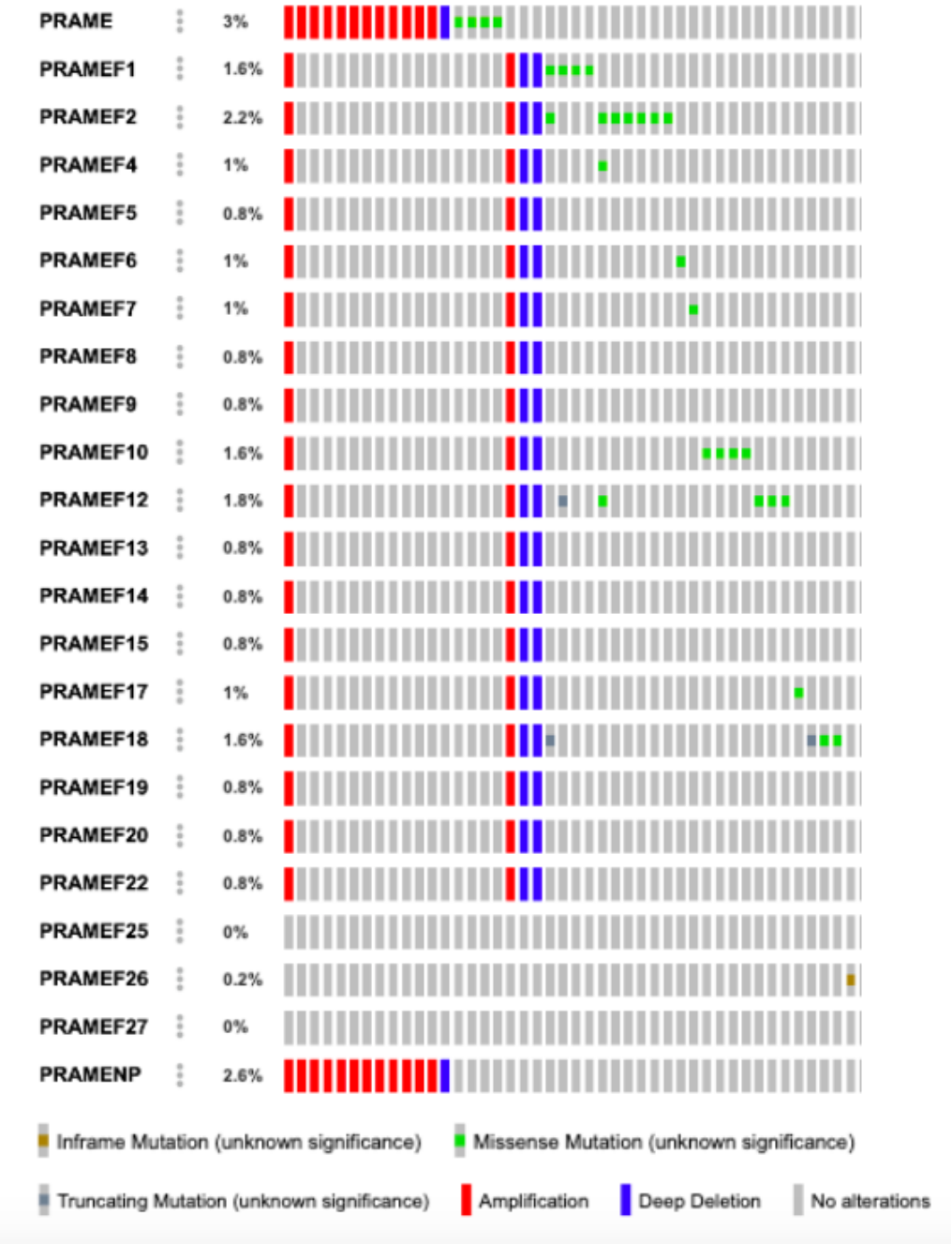

Figure 1 The oncoprint data depicting different types of gene alterations in the *PRAME* family of genes.

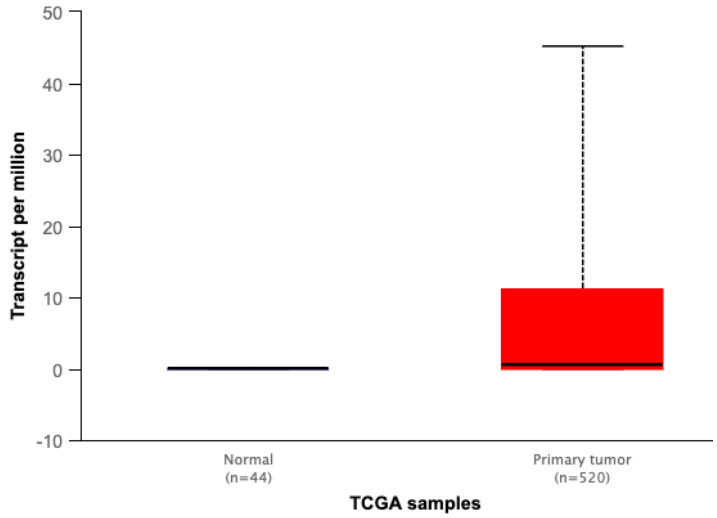

Figure 2 represents the expression of the *PRAME* gene in the primary tumor of HNSCC patients relative to normal samples. X axis represents TCGA sample dataset and Y axis represents the *PRAME* gene expression (transcript per million). The p value was found to be 1.11×10^{-16}, where a p value <0.05 was considered to be significant.

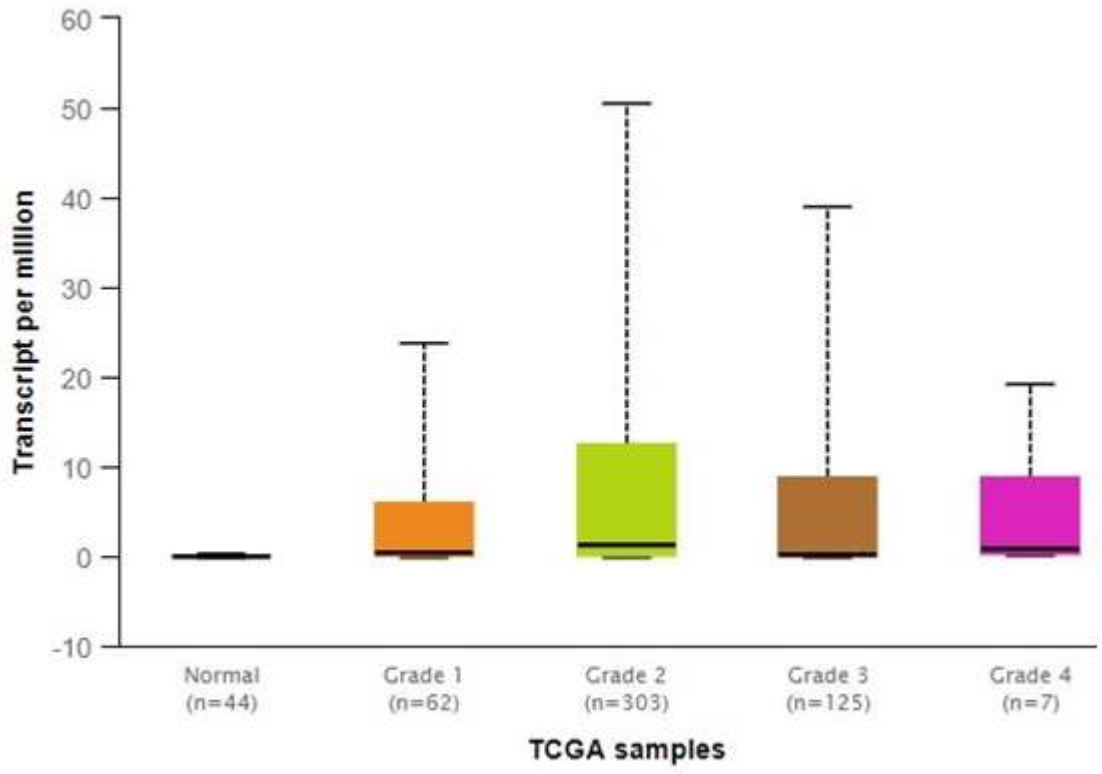

Figure 3 Box-whisker plot representing the differential gene expression pattern of the *PRAME* gene across different tumor grades. X axis represents the different grades of HNSCC samples from the TCGA data set and Y axis represents the *PRAME* gene expression in HNSC in transcript per million (TPM). A significant difference in the gene expression profile was observed between normal vs grade 1 ($p = 8.6 \times 10^{-4}$), normal vs grade 2 ($p = < 10^{-12}$) and normal vs grade 3 (3.125×10^{-8}). A p value less than 0.05 is considered to be significant.

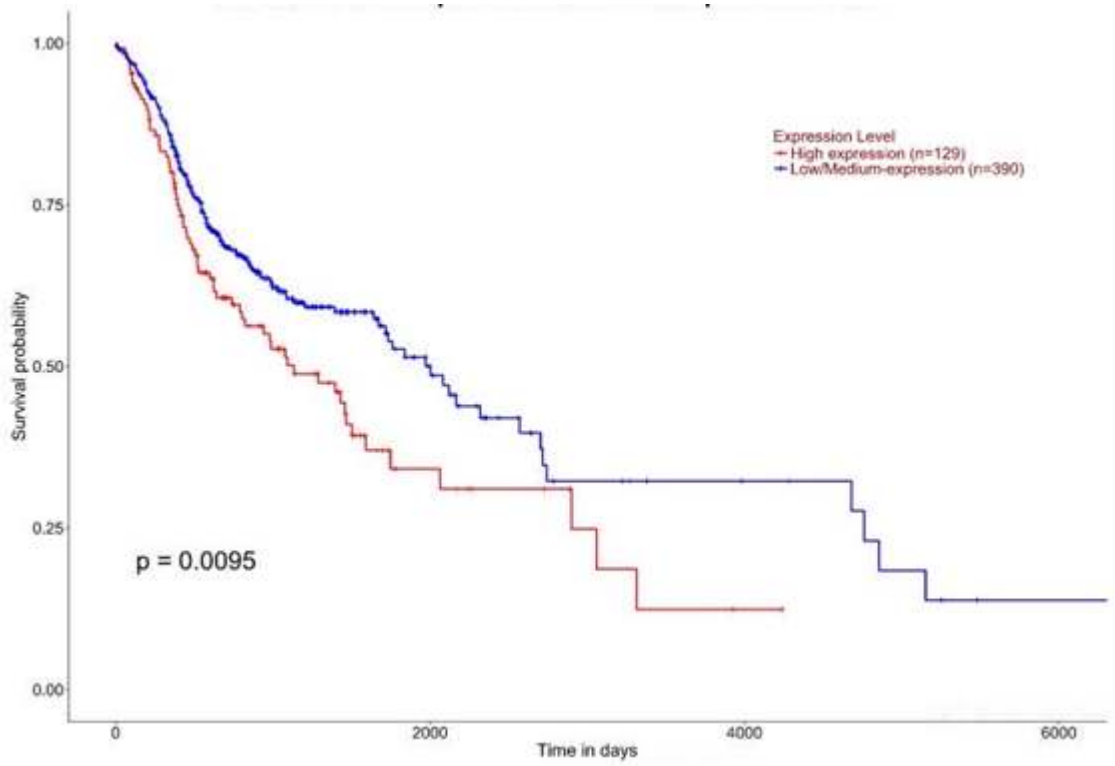

Figure 4 Kalplan-Meier plot showing the association of altered PRAME expression with HNSC patient's survival. The x - axis represents time in days and y - axis denotes the survival probability in HNSC patients. The red line corresponds to high level expression and the blue line represents low/medium level expression. A significant association was observed between high and low/medium level expression of PRAME (p = 0.0095). A p value less than 0.05 is considered to be significant.

Table 1: Demographic details of patients analysed in the present study (as obtained from the

cBioportal site)

Gender	**Male (n = 386)** **Female (n = 142)**
Mutation count	6-3181
Diagnosis age	19-90 years
Smoking status	Smokers: 515 Data not available: 12 Unknown: 1
Alcohol history	Yes – 352 No – 165 Data not available: 11
Neoplasm Histologic grade	Grade 1: 63 Grade 2: 311 Grade 3: 125 Grade 4: 7 Grade GX: 18 Data not available: 4
Race category	White: 452 African: 48 Asian: 11 American Indian or Alaska native: 2 Data not available: 15

Table 2: Frequency and type of genetic alteration in the *PRAME* family of genes

Gene	Protein	Alteration	Cytogenetic location	Percentage of alteration	Variant allele frequency in tumor sample	gnomAD frequency data
PRAME	Preferentially expressed Antigen in Melanoma	Amplification Deep deletion L119R Q287P L313R R125Q	22q11.22	3	- - 0.12 0.30 0.50 0.06	- - Novel Novel Novel
PRAMEF1	PRAME family member 1	Amplification Deep deletion V171I E338K T72K N297K	1p36.21	1.6	- - 0.23 0.23 0.38 0.03	- - Novel Novel rs149382773 Novel

PRAMEF2	PRAME family member 2	Amplification Deep deletion Y194H H268R A114P E11Q L313I R174M T381A	1p36.21	2.2	- - 0.02 0.19 0.39 0.19 0.19 0.04 0.06	 Novel Novel Novel Novel Novel Novel Novel
PRAMEF4	PRAME family member 4	Amplification Deep deletion P418L	1p36.21	1	- - 0.02	 rs753793229
PRAMEF5	PRAME family member 5	Amplification Deep deletion	1p36.21	0.8	-	-
PRAMEF6	PRAME family member 6	Amplification Deep deletion V135E	1p36.21	1	- - 0.10	- - Novel
PRAMEF7	PRAME family member 7	Amplification Deep deletion S317N	1p36.21	1	- - 0.13	- - rs779669158
PRAMEF8	PRAME family member 8	Amplification Deep deletion	1p36.21	0.8	- -	- -

PRAMEF9	PRAME family member 9	Amplification Deep deletion	1p36.21	0.8	- -	- -
PRAMEF10	PRAME family member 10	Amplification Deep deletion Q270L M46R L266P R96S	1p36.21	1.6	- - 0.20 0.61 0.23 0.10	- - Novel rs1167071023 Novel Novel
PRAMEF12	PRAME family member 12	Amplification Deep deletion R94C Q4* S307W P238L S128I	1p36.21	1.8	- - 0.27 0.25 0.26 0.21 0.29	- - rs752095583 rs757917825 Novel Novel Novel
PRAMEF13	PRAME family member 13	Amplification Deep deletion	1p36.21	0.8	- -	- -
PRAMEF14	PRAME family member 14	Amplification Deep deletion	1p36.21	0.8	- -	- -

PRAMEF15	PRAME family member 15	Amplification Deep deletion	1p36.21	0.8	- -	- -
PRAMEF17	PRAME family member 17	Amplification Deep deletion S117A	1p36.21	1	- - 0.15	- - Novel
PRAMEF18	PRAME family member 18	Amplification Deep deletion N448Tfs*? N448Qfs*19 L354V L373M	1p36.21	1.6	- - 0.33 0.36 0.28 0.03	- - Novel Novel rs1384433084 Novel
PRAMEF19	PRAME family member 19	Amplification Deep deletion	1p36.21	0.8	- -	- -
PRAMEF20	PRAME family member 20	Amplification Deep deletion	1p36.21	0.8	- -	- -
PRAMEF22	PRAME family member 22	Amplification Deep deletion	1p36.21	0.8	- -	- -
PRAMEF25	PRAME family member 22	Amplification Deep deletion	1p36.21	0	- -	- -

PRAMEF26	PRAME family member 26	K159del	1p36.21	0.2	0.14	Novel
PRAMEF27	PRAME family member 22	Amplification Deep deletion	1p36.21	0	- -	- -
PRAMENP	PRAME N-Terminal like, Pseudogene	Amplification Deep deletion	22q11.22	2.6	- -	- -

Table 3: Protein stability and pathogenesis of variants identified in *PRAME* family of genes

Gene	Alteration	IMutant Prediction	IMutant Score	PROVEAN Prediction	PROVEAN Score
PRAME	L119R	Decrease	-0.95	Deleterious	-5.964
	Q287P	Decrease	-1.13	Deleterious	-5.407
	L313R	Decrease	-1.30	Deleterious	-5.843
	R125Q	Decrease	-1.07	Deleterious	-2.571
PRAMEF1	V171I	Decrease	-0.06	Neutral	-0.844
	E338K	Increase	0.15	Neutral	-2.075
	T72K	Decrease	-0.32	Neutral	-1.305
	N297K	Decrease	-0.60	Neutral	-1.822

PRAMEF2	Y194H	Decrease	- 0.76	Neutral	1.041
	H268R	Increase	0.36	Deleterious	-4.565
	A114P	Decrease	-1.40	Neutral	-1.356
	E11Q	Increase	0.45	Neutral	-1.601
	L313I	Increase	0.51	Neutral	-1.854
	R174M	Decrease	-0.57	Deleterious	-5.405
	T381A	Decrease	-0.17	Deleterious	-3.315
PRAMEF4	P418L	Decrease	-0.91	Deleterious	-9.315
PRAMEF6	V135E	Decrease	-1.35	Deleterious	-2.89
PRAMEF7	S317N	Decrease	-2.18	Neutral	0.691
PRAMEF10	Q270L	Decrease	-0.37	Deleterious	-5.046
	M46R	Increase	0.02	Neutral	2.204
	L266P	Decrease	-0.88	Deleterious	-5.697
	R96S	Decrease	-1.55	Deleterious	-5.633
PRAMEF12	R94C	Decrease	-0.99	Deleterious	-4.786
	S307W	Increase	0.25	Deleterious	-5.728
	S128I	Increase	0.80	Neutral	-2.302
PRAMEF17	A117S	Increase	0.09	Neutral	-2.11
PRAMEF18	L354V	Increase	0.97	Deleterious	-2.818
	L373M	Decrease	-0.07	Neutral	-1.943