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Computational analysis of *CDH1* missense mutations in the cause of hereditary diffuse gastric cancer

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Article Info

Abstract

In this present study, we computationally identified the germline missense mutation in the E-cadherin (*CDH1*) gene causing hereditary diffuse gastric cancer (HDGC). The analysis was initiated with SIFT followed by PolyPhen and I-Mutant2.0 programs with the help of 68 *CDH1* variants retrieved from dbSNP. The analysis indicates that 10 variants such as P201R, A298T, E336D, C695R, N751K, Y755C, D768N, G879S, D882N and R169H were commonly found to be less stable and damaging by SIFT, PolyPhen and I-Mutant2.0 programs. Furthermore, SNPs and GO was used to predict the disease related mutations from the protein sequence. Finally, the affinities for the cetuximab with *CDH1* variants were examined by using molecular docking algorithm. The result showed that P201R, A298T, E336D and R169H variants were found to be highly significant than the other mutations considered in our analysis. We sincerely hope that these findings certainly helpful for the experimental biologist working in HDGC drug development.

Introduction

E-Cadherin (*CDH1*) is cell-cell adhesion molecule localized in adherens junctions. The function involves polarity, cell differentiation, tissue integrity and regulating signal transduction pathways (Qian et al., 2004). The extracellular portion of the protein mediates homophilic cellular interactions, and intracellular part provides a link to the actin cytoskeleton through β-catenins, a multifunctional protein associated with *CDH1* gene (Keller et al., 1999).

Loss of function or expression of E-cadherin increases the invasion and metastasis of tumors. It is being called as "Suppression of invasion" gene. It includes dysfunction of cell-cell adhesion, loss of tissue integrity, morphological changes, Loss of heterozygosity (LOH) and increased proliferation. The presence of *CDH1* in chromosome 16q22.1 was confirmed by the researchers (Pećina, 2003). The literature evidences available suggest that missense mutation in the *CDH1* gene might causes gastric, breast, colorectal, thyroid, endo-metrium and ovarian cancers (Berx et al., 1998). Moreover, gastric cancer is more predominant than the other cancer types especially caused by the missense mutation in *CDH1* gene (Corso et al., 2012). It is the 4th most common cancer and 2nd most cancer death worldwide (Zhang et al., 2006). The germline missense mutations of E-cadherin resulting in E-cadherin inactivation was identified as the supreme importance for the Hereditary Diffuse Gastric Cancer (HDGC) (Kim et al., 2000).

The majority of the families with autosomal dominant gastric cancer susceptibility have HDGC (Oliveira et al., 2004; Brooks et al., 2004; Kaurah et al., 2007). E-cadherin deficiency provides an obvious explanation for the diffuse, scattered growth of HDGC tumours, as the protein is the central component of epithelial cell-to-cell adhesion junctions and as such is required for the integrity of epithelial layers (More et al., 2007). In gastric cancer a series of trials have produced evidence that chemotherapy increases survival. There are so many drugs available for the chemotherapy treatment

(Nishiyama and Wada, 2009; Graziano et al., 2004; Zhang et al., 2013). However, the cetuximab proved to be effective drug for the treatment of HDGC (Cunningham et al., 2004). E-cadherin expression increases the sensitivity to cetuximab in gastric cancer cell lines. Cetuximab monotherapy has the improved treatment outcome compare to other chemotherapy drugs (Heindl et al., 2012). By stimulating an immune system mediated anti tumour response, cetuximab inhibits cancer-cell proliferation, angiogenic growth factor production and tumor-induced angiogenesis, and cancer cell invasion.

In gastric cancer treatment, cetuximab is over expressed with the target cells (Lordick et al., 2010). The literature evidences indicates that mutation in E-cadherin leads to the improper binding of cetuximab and leads to the cetuximab resistance. Therefore, monitoring the cetuximab resistance is a key area for the treatment of HDGC. This would be certainly helpful for the development of long acting drug molecule. Hence, in this present study, we identified detrimental missense mutations in E-cadherin using different genomic algorithms. Subsequently, the sensitivity of cetuximab with mutated cells was also examined by docking analysis.

Materials and Methods

The SNPs (Single Nucleotide Polymorphisms) and their associated information for *CDH1* gene were obtained from dbSNP database (http://www.ncbi.nlm.nih.gov/ SNP/) for our computational analysis. The protein sequence corresponds to E-cadherin were obtained from uniprot (http://www.uniprot.org/). The 3D Structure of *CDH1* protein obtained from Swissmodel workspace (Arnold et al., 2006) (http:// swissmodel.expasy.org/). The mutant structures were generated by using SPDB Viewer package. The 3D structure of the cetuximab was retrieved from Protein Data Bank (Berman et al., 2000) (http:// www.rcsb.org/) for the molecular docking analysis.

Investigations of structural and functional consequences of coding nsSNPs by computational analysis

The SNP occurring in the protein coding region normally leads to the deleterious consequences in its 3D structure and hence may prone to disease-associated phenomena. In the present study, we used the genomic tools such as SIFT (Ng and Henikoff, 2003), PolyPhen-2 (Ramensky et al., 2002), I-Mutant2.0 (Capriotti et al., 2005) and SNPs & Go (Calabrese et al., 2009) to detect the deleterious coding nsSNPs, and FireDock (Mashiach et al., 2008) to calculate the binding free energy.

Tolerance analysis of missense mutations by SIFT

SIFT (Sorting Intolerant From Tolerant) is a sequence homology based tool available at http:// www.blocks.fhcrc.org/sift/SIFT.html. It presumes that important amino acids will be conserved in the protein family. Thus, changes at well conserved positions tend to be predicted as deleterious. We submitted the query in the form of SNP IDs or as protein sequences. The underlying principle of this program is that SIFT takes a query sequence and uses multiple alignment informa-tion to predict tolerated and deleterious substitutions for every position of the query sequence. SIFT is a multistep procedure that, given a protein sequence, i) searches for similar sequences, ii) chooses closely related sequences that may share similar functions, iii) obtains the multiple alignment of the chosen sequences, and iv) calculates normalized probabilities for all possible substitutions at each position from the align-ment. Substitutions at each position which normalized probabilities less than a chosen cutoff are predicted to be deleterious and those greater than or equal to the cutoff are predicted to be tolerated (Ng and Henikoff, 2003). The cutoff value in the SIFT program is a tole-rance index of ≥0.05. The higher the tolerance index, the less function impact a particular amino acid substi-tution is likely to have.

Prediction by PolyPhen-2

The structural level analysis of coding nsSNPs at is considered to be very important to understand the functional activity of the protein. In the present study, structural level analysis was performed with the aid of PolyPhen-2 (Ramensky et al., 2002), which is available at http://coot.embl.de/Polyphen/. Input options for the PolyPhen-2 program are protein sequence or accession number together with sequence position with two amino acid variants. We submitted the query in the form of protein sequence with mutational position and two amino acid variants. Sequence based characteri-zation of the substitution site, profile analysis of homo-logous sequences, and mapping of substitution site to a known protein three dimensional structure are the parameters taken into account by the PolyPhen-2 prog-ram to calculate the score. It calculates PSIC scores for each of the two variants and then computes the difference between them. The higher the PSIC score difference, the higher is the possible functional impact of a particular amino acid substitution.

Stability analysis with I-Mutant2.0

I-Mutant2.0 is a support vector machine (SVM) based tool for the automatic prediction of protein stability changes caused by single point mutations. The predic-tions were performed starting either from the protein structure or, more importantly, from the protein sequence (Capriotti et al., 2005). The output files show the predicted free energy change value ($\Delta\Delta G$), which was calculated from the unfolding Gibbs free energy value of the mutated protein minus the unfolding Gibbs free energy value of the native protein (kcal/mol). Positive $\Delta\Delta G$ values meant that the mutated protein has higher stability and negative values are the indication of lesser stability.

Prediction of disease related mutations using SNPs &

GO

Furthermore, we have used Single Nucleotide Polymorphism Database (SNPs) & Gene Ontology (GO) are support vector machine (SVM) based accurate methods used to predict the disease related mutations from protein sequences with a scoring accuracy of 82% and Matthews correlation coefficient of 0.63 (Calabrese et al., 2009). The FASTA sequence of whole protein is considered to be an input option and output will be the prediction results based on the discrimination among disease related and neutral variations of protein sequence. The RI (Reliability Index) higher than 5 reveals the disease related effect of mutation on the parent protein function.

Homology modelling and RMSD analysis

The sequence version of the human E-cadherin protein was retrieved from Swiss-prot (http://www.expasy.ch/sprot/). Then a BLAST (http://www.ncbi.nlm.nih.gov/blast/) sequence analysis was performed against the whole PDB to select the template that could be used to generate the model of E-cadherin. Subsequently, the three dimensional structure of the model for the E-cadherin was generated by the homology modeling software from the Swissmodel workspace (http://swissmodel.expasy.org/). Furthermore, the mutated model structure was generated by means of SwissPDB viewer. We used conjugate gradient method for optimizing the 3D structures. The deviation between the two structures was evaluated by their Root Mean Square Deviation (RMSD) analysis.

Molecular docking studies

The FireDock (Mashiach et al., 2008), Fast Interaction REfinement in molecular docking algorithm was used to calculate the binding affinity of cetuximab with E-cadherin. This program is available at http://www.bioinfo3d.cs.tau.ac.il/FireDock/index.html. The method simultaneously targets the problem of flexibility and scoring of solutions produced by fast rigid-body docking algorithms. Given a set of up to 1000 potential docking candidates, FireDock refines and scores them according to an energy function, spending about 3.5 seconds per candidate solution. The candidate solutions for FireDock can be generated by rigid-body docking methods, such as PatchDock, FFT-based methods such as ZDOCK, GRAMM-X, Hex, ClusPro etc. The output is a table of ranked global energy values. The refined complex structure is generated for up to 100 low-energy candidates.

Results and Discussion

There are 68 missense mutations, namely M203V, R29Q, V153I, T211P, W483S, N155S, R162W, R28W, V138M, A80T, R169H, M177T, K69T, C306R, E336D, N674Y, P597S, R224H, K182N, T395A, M282I, G879S, D805N, K184I, R224C, D882N, D443N, E410K, Y755C, A401D, N666H, E702K, S543F, N622S, P201R, I535V, K381N, E864K, A298T, V132I, V392I, D768N, P30T, A788V, A408V, A709S, A634V, S838G, L711V, T340A, V574F, R124H, D676N, T506A, V242I, T340M, D498E, V473I, D72N, V832M, L478P, A592T, E880K, I393N, N751K, A617T, C695R, L630V were examined in this work retrieved from dbSNP (Smigielski et al., 2000).

The mutations were independently submitted into SIFT program to check its tolerance index (Ng and Henikoff, 2003). Among the 68 variants, 24 variants found to be deleterious having the tolerance index score of ≤0.05. The result is shown in Table I. We observer that, Out of 24 variants 8 variants were having highly deleterious tolerance score 0. Six variants were having tolerance index score of 0.01, four variants were having tolerance index score of 0.02, one variant had a tolerance index score of 0.03, four variants were having tolerance index score of 0.04 and one variant had a tolerance index score of 0.05.

Protein sequence with mutational position and amino acid variants associated with 68 single point mutants, used in this work were submitted as input to the PolyPhen program (Ramensky et al., 2002) and results were shown in Table I. A PSIC score difference of 1.5 and above was considered to be damaging. Out of 68 variants 23 variants were considered to be damaging by PolyPhen program. Interestingly 13 variants namely, R162W, R169H, N674Y, E336D, A298T, P201R, C695R, G879S, D882N, Y755C, D768N, I393N and N751K were considered to be damaging by PolyPhen also were seen to be deleterious according to the SIFT program.

To further probe this behaviour, we used I-Mutant 2.0 program for our analysis. This program predicts the stability to the protein structure by means of $\Delta\Delta G$ value. Out of 68 variants, we obtained 53 variants found to be less stable from the I-Mutant 2.0 Program (Capriotti et al., 2005) as shown in Table I. It is interesting to observe that 5 variants showed a $\Delta\Delta G$ value of >-3.0 kcal/mol. The other 7 variants were showed a $\Delta\Delta G$ value of >-2.0 kcal/mol. The other 16 variants showed a $\Delta\Delta G$ value of >-1.0 kcal/mol. The remaining 25 variants showed a $\Delta\Delta G$ value of <-1.0 kcal/mol as depicted in Table I. Out of 53 variants which showed a negative $\Delta\Delta G$, 4 variants namely, E864K, E702K, E880K and E410K changed their amino acid from negatively charged to positively charged amino acid; 10 variants such as P597S, G879S, A709S, M177T, A80T, A298T, A617T, P30T, A592T and I393N changed from non-polar to polar amino acid; 4 variants, N666H, C306R, N751K and C695R changed from polar to positively charged amino acid; 5 variants, T340M, T340A, T506A, T211P, T395A and S838G changed from polar to non-polar amino acid. S543F and N674Y changed from polar to aromatic amino acid. 2 variants, K184I and K69T changed from positively charged to

Table I				
List of variants that were predicted to be functionally significant by SIFT, PolyPhen, I-Mutant 2.0				
Amino acid change	Tolerance index	PSIC SD	Prediction	DDG (kcal/mol)
M203V	0.53	0.002	Benign	-0.52
R29Q	0.01	0.697	Possibly Damaging	-0.65
V153I	0.35	0.012	Benign	-0.5
T211P	0.03	0.725	Possibly Damaging	-3.18
W483S	1	0	Benign	-0.86
N155S	0.78	0	Benign	0.03
R162W	0.01	0.997	Probably Damaging	0.03
R28W	0	0	Benign	-2.28
V138M	0.18	0.156	Benign	0.82
A80T	0.06	0.005	Nil	-1.19
R169H	0	0.984	Probably Damaging	-2.35
M177T	0.61	0	Benign	-3.15
K69T	0.25	0.939	Possibly Damaging	-1.2
C306R	0.01	0.004	Benign	0.22
E336D	0.04	0.963	Probably Damaging	-1.07
N674Y	0.01	0.99	Probably Damaging	1.22
P597S	0.69	0.326	Benign	-0.62
R224H	0.17	0	Benign	-2.72
K182N	0.28	0.103	Benign	-1.68
T395A	0.52	0.008	Benign	-0.47
M282I	0.14	0.997	Probably Damaging	0.57
G879S	0.02	1	Probably Damaging	-2.1
D805N	0.09	1	Probably Damaging	-0.34
K184I	0.14	0.984	Probably Damaging	-0.65
R224C	0.04	0.657	Possibly Damaging	-2.42
D882N	0	1	Probably Damaging	-1.29
D433N	0.45	0.023	Benign	-3.47
E410K	0.94	0	Benign	-2.3
Y755C	0	1	Probably Damaging	-1.04
A401D	0.63	0.003	Benign	-0.52
N666H	0.63	0.004	Benign	-0.38
E702K	0.56	0.014	Benign	-0.65
S543F	0.08	0.011	Benign	0.16
N622S	0.01	0.901	Possibly Damaging	-0.55
P201R	0	1	Probably Damaging	-0.05
I535V	0.22	0.001	Benign	-0.69
K381N	0.39	0.001	Benign	-1.82
E864K	0.28	0.867	Possibly Damaging	-0.6
A298T	0.02	0.999	Probably Damaging	-2.39
V132I	0.25	0.322	Benign	0.95
V392I	0.33	0.001	Benign	1
D768N	0	1	Probably Damaging	-0.18
P30T	0.12	0.986	Probably Damaging	-0.56
A788V	0.23	0.722	Possibly Damaging	-1.47
A408V	0.3	0.995	Probably Damaging	-0.77
A709S	0.31	0.995	Probably Damaging	-0.27
A634V	0.52	0.051	Benign	-0.19
S838G	0.67	0.022	Benign	-1.7
L711V	0.02	0.864	Possibly Damaging	-0.05
T340A	0.62	0.001	Benign	-3.58
V574F	0.02	0.067	Benign	-0.55
R124H	0.14	0	Benign	-2.47
D676N	0.25	0.752	Possibly Damaging	-1.98
T506A	1	0	Benign	0.28
V242I	0.35	0.006	Benign	-0.45
T340M	0.12	0.085	Benign	-1.09
D498E	0.04	0.903	Possibly Damaging	-0.16

Table I				
(Continued)				
Amino acid change	Tolerance index	PSIC SD	Prediction	DDG (kcal/mol)
V473I	0.13	0.977	Probably Damaging	-2.03
D72N	0.19	0.994	Probably Damaging	-1.77
V832M	0.06	1	Probably Damaging	-0.76
L478P	0.27	0.507	Possibly Damaging	-1.59
A592T	0.04	0.492	Possibly Damaging	-0.85
E880K	0.01	0.495	Possibly Damaging	-1.15
I393N	0	0.997	Probably Damaging	0.09
N751K	0	1	Probably Damaging	0.99
A617T	0.67	0.04	Benign	-3.9
C695R	0.05	1	Probably Damaging	-1.31
L630V	0.18	0.998	Probably Damaging	-1.07

Table II		
Disease related prediction by SNPs & GO		
Variants	RI	Prediction
E336D	6	Disease
A298T	7	Disease
P201R	6	Disease
C695R	1	Neutral
G879S	1	Neutral
D882N	4	Neutral
Y755C	1	Disease
R169H	6	Disease
D768N	1	Neutral
N751K	1	Neutral
RI<5 predicted to be neutral and RI>5 predicted to be disease		

and Y755C changed from aromatic to polar amino acid. Finally, the variants such as A401D, P201R and V574F changed from non-polar to negatively charged, positively charged and aromatic amino acid, respectively. It is also to be noted that M203V, M282I, A408V, A634V, A788V, V242I, V153I, V392I, V132I, V473I, L711V, L630V, L478P, I535V, V138M and V832M variants retained non-polar amino acid, N155S and N622S variants retains polar amino acid, E336D and D498E, variants retained negatively charged amino acid, R169H and R124H, variants retained positively charged amino acid property were found to be less stable by I-Mutant 2.0. Most importantly, 18 variants were considered to be damaging by PolyPhen program were also seen to be deleterious according to I-Mutant2.0 program. The above point portrays that preserving amino acid physico-chemical properties does not necessary result in harmless mutation. Indeed considering only amino acid substitution based on physico-chemical properties could not be able to identify the detrimental effect rather than considering the sequence conservation along with the above said properties could have more

non-polar amino acid; R29Q, R224H, K182N, R224C and K381N changed from positively charged to polar amino acid; R162W and R28W changed from positively charged to aromatic amino acid. 6 variants, D805N, D882N, D443N, D768N, D676N and D72N changed from negatively charged to polar amino acid; W483S
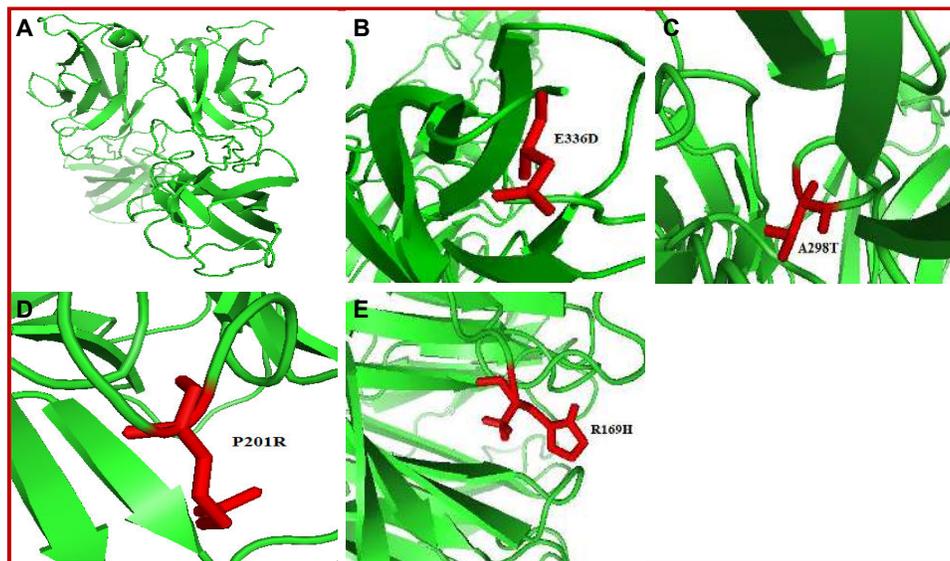
Figure 1: PyMol view of modelled structures (A) native structure (B) mutant (E336D) structure (C) mutant (A298T) structure (D) mutant (P201R) structure (E) mutant (R169H) structure

Table III	
RMSD for native and mutant structures	
Structure	RMSD (Å)
Native 1	0.000
M1(E336D)	0.015
M2(A298T)	0.105
M3(P201R)	3.617
M4(R169H)	0.028

advantages and reliable to find out the detrimental effect of missense mutations (Teng et al., 2009).

We rationally considered the 10 potential detrimental point mutations such as E336D, A298T, P201R, C695R, G879S, D882N, Y755C, R169H, D768N and N751K for further course of investigation. They were commonly found to be less stable, deleterious and damaging by the I-Mutant2.0, SIFT and PolyPhen programs, respectively. It was interestingly to note that among these 10 variants, E336D and A298T showed very good agreement with experimental observation performed
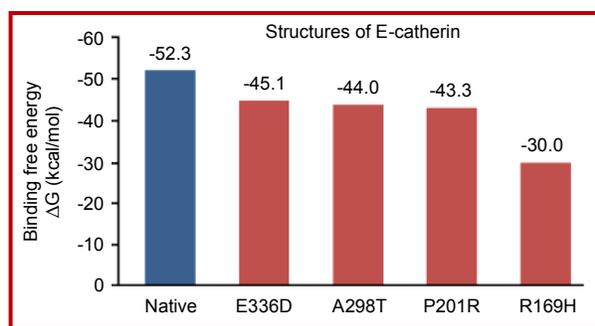
Figure 2: Estimation of free energy of binding, DG, (kcal/mol) using FireDock

elsewhere (Berx et al., 1998; Corso et al., 2012; Oliveira et al., 2004; Brooks et al., 2004; Kaurah et al., 2007).

In order to predict the human disease related single point protein mutations we used SNPs and GO program (Calabrese et al., 2009) to predict a particular variant is disease related or neutral. Among the 10 detrimental missense mutations 4 variants namely, E336D, A298T, 201R and R169H found to be diseased and the remaining 6 variants predicted to be neutral by SNPs and GO program. The result is shown in Table II. We observed that, out of 10 variants, 4 variants were aving RI of >5 reveal the disease related effect and the remaining 6 variants were having RI of <5 indicate relatively the neutral effect.

The four detrimental structure of *CDH1* were generated by means of Swiss model program. The mutant structures (E336D, A298T, P201R and R169H) were generated by SwissPDB viewer. The PyMol view of the modelled structures of E-cadherin is shown in Figure 1.

In order to find out the deviation between the two

structures, we superimposed the energy refined native structure with all the energy refined mutant structures to get RMSD. The higher the RMSD value, the more is the deviation between the native and the mutant structure, which in turn changes their binding efficiency with inhibitors due to deviation in 3D space of the binding residues of *CDH1* gene. Table III shows the RMSD for native structure with all the mutant modelled structures. The value is of 0.015 Å, 0.105 Å, 3.617Å and 0.028 Å for the E336D, A298T, P201R and R169H structures respectively.

Finally, the molecular docking studies were performed to confirm the functional impact of the amino acid mutation. Cetuximab (PDB ID: 1yy8) structure was retrieved from PDB. It is docked with native and mutant (E336D, A298T, P201R and R169H) structures of E-cadherin to understand the binding affinity. Docking was performed using the FireDock program (Mashiach et al., 2008). The result is shown from Figure 2. The analysis indicates the affinity for cetuximab for native *CDH1* was found to be -52.3 kcal/mol, whereas with the mutants, the DG was found to be in the ranges –30.0 to -45 kcal/mol. It can be seen from Figure 2, the mutants established lesser binding affinity with cetuximab than the native type protein. These data clearly portray that mutation in the E-cadherin structure leads to the resistance for cetuximab. This is the clear evidence of the deleterious effect of missense mutations such as E336D, A298T, P201R and R169H lesser. Hence, we conclude that these variants should also consider for the design of drug for the treatment of HDGC.

The mutations of *CDH1* namely E336D and A298T were proved more deleterious effect to the structural stability and its function of the E-catherin. In this work, we also found quite a few other drug-resistant mutations by computational approach. We believe that our observations have critical implications for the understanding of *CDH1* associated missense mutations and also for the development of novel therapies for this disease.

Acknowledgement

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