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THE INFLUENCE OF EXON 7 PHE389LEU POLYMORPHISM ON P120 CATENIN INTERACTIONS WITH E-CADHERIN AND THREE-DIMENSIONAL MODEL REBUILT

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Abstract – The significance of endothelial P120 catenin (P120ctn) activity has been recognized for many years, however it was only recently that the complicated regulation of this constitutively expressed enzyme in endothelial cells was identified. A critical component of the P120ctn regulatory cycle in endothelial cells is its intracellular localization to caveolae. The caveolar coordination of P120ctn, more specifically its interaction with E-cadherin plays a major role in normal endothelial P120ctn activity and vascular bioavailability of nitric oxide. We have recently shown that the presence of P120ctn exon 7 Phe389Leu polymorphism caused diminished shear which was dependent catenin activation, was less extensively associated with caveolae, and had a decreased degree of interaction with E-cadherin. Here, we carried out preliminary investigations to identify possible mechanisms of the genotype-dependent endothelial cell responses we observed in our previous investigations. Through this approach we tested the hypothesis that computer simulations could provide insights regarding the contribution of this single nucleotide polymorphism to regulation of the P120ctn isoform. We observed that in the Phe/Leu and Leu/Leu mutant genotypes, the amount of P120ctn associated with E-cadherin was significantly lower. Additionally, we have shown, using a theoretical computational model, that mutation of an amino acid at position 389 might affect the protein-protein interactions and localization of the P120ctn protein. These alterations might also affect the protein function and explain the enhanced disease risk associated with the presence of Phe389Leu polymorphism in the P120ctn protein.

Key words: P120catenin, E-cadherin, gene polymorphism, Three-dimensional model.

INTRODUCTION

The vascular endothelium is highly metabolically active and plays a key role in vascular homeostasis through the release of a variety of substances, including the catenin (7). The actions of catenin can be altered severely under various conditions of stress, such as hypoxia, oxidants, reactive nitrogen species, and shear stress (19). Diminished catenin availability has been associated with several settings of endothelial impairment, including acute hypoxic or hyperoxicorgan injury and chronic forms of vascular disease (12). P120catenin (P120ctn), a member of catenin family is important for maintenance of vascular remodeling and angiogenesis (6,11,13).

A critical component of the P120ctn regulatory cycle in endothelial cells is its intracellular localization to cadherin (10). Cadherin are rich membrane microdomains in the plasma membrane that provide intracellular coordination of many membrane. Associated proteins an d putatively function as regulators of cholesterol transport, endocytosis, and signal transduction (8,14). In endothelial cells, E-cadherin, a 100-kDa protein that coats the cytoplasmic surface of this specialized microdomain, is the major protein constituent of cadherin and is thought to hold or “store” P120ctn in an inactive state, wherein phosphorylation causes dissociation and P120ctn catalytic activation(2,17). The cadherinal coordination of P120ctn, more specifically its interaction with E-cadherin, plays a major role in normal endothelial P120ctn activity and vascular bioavailability of catenin and is a key controller of shear-dependent catenin release (15).

Several specific allelic variations of the P120ctn gene are known to date, with few of these having been linked to increased vascular

disease risk, and nobody reported a single nucleotide polymorphism (SNP) (C>A) at position 1167, leading to the sequence change Phe389Leu was associated with vascular disease. Using a "candidate gene'' approach, in which patient genotypes are related to the incidence of a disease and/or clinical outcome to test for statistically significant associations (parametric and/or non-parametric linkage analyses), but few reports have indicated that the P120ctn Phe389Leu polymorphism is associated with increased occurrence of vascular disease, and the exact biological consequences of this specific variation are not identified.

Given that cadherinal associations have been recognized as important for normal P120ctn performance in endothelial cells, and the established importance of this variation for disease risk in humans. We recently investigated the impact of the P120ctn G1u389Leu polymorphism on endothelial cell responses to shear stress and the localization of cadherin. We found initial evidence that the altered enzyme, when in its native endothelial cell environment, has diminished shear-dependent catenin activation, is less extensively associated with cadherin, and has a decreased degree of interaction with E-cadherin (20). Our preliminary findings using protein biochemistry techniques thus suggested that the Phe389Leu variation might influence protein-protein interactions. In this project we carried out preliminary computer modeling to further investigate possible mechanisms of the genotype-dependent endothelial cell responses we observed in our previous investigations (20). Through this approach we tested the hypothesis that computer simulations could provide insights regarding the contribution of this SNP to regulation of the P120ctn isoform.

MATERIALS AND METHODS

P120ctn genotyping

Human umbilical vein endothelial cells purchased from American Type Cell Culture Collection (ATCC, Rockville, MD) were screened for P120ctn genotype using polymerase chain reaction-based DNA amplification followed by restriction enzyme digestion as described previously(20). Genomic DNA was extracted from cell lines using Genomic DNA Purification Kit MagExtractor® according to the manufacture's protocol (ToYoBo, Japan). The 1167C/A polymorphism of P120ctn were examined by PCR-restriction fragment length polymorphism (RFLP) and PCR-single-strand conformation polymorphism (SSCP), respectively. A 190 bp fragment containing the −160 site was amplified with the sense primer 5'-tcccaggtcttagtgagcca-3' and the corresponding antisense primer 5'-acgactaaccgacaccgg-3'. PCR reaction was initiated with a denaturation for 5 min at 95 ℃; followed by 35 cycles of 95℃ for 30 s, 60℃ for 30 s, and 72 ℃ for 30 s; and a final extension at 72℃ for 5 min. The PCR products containing the A-allele were expected to be cleaved into two fragments (110 and 80 bp) after digestion by the enzyme Hincll at 37 ℃ for 16 h (New England Biolabs, USA). Negative and positive controls were set in parallel to ensure that PCR products were not polluted and Hincll worked correctly. The SSCP experiment with the primers and PCR conditions reported previously was performed on a Dcode Universal Mutation Detection System (Bio-Rad) . In brief, after denaturing at 95℃ for 10 min and snap-cooling on ice for 5 min, the PCR products of 10 μl were detected by 8% PAGE in 1× TBE running buffer at 15W for 8 h. Then the gels were stained with ethidium bromide solution and visualized under UV illumination. The PCR products of typical samples were separated on agarose gels, and the target bands were excised and purified with the QIAquick Gel Extraction Kit (Qiagen, Germany), and then sequenced directly.

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Cell lysis and immunoprecipitation

After washing three times with ice-cold phosphate-buffered saline, cells were lysed in NP-40 lysis buffer containing 1% NP-40, 50 mmol/L Tris, PH 7.5, 150 mmol/L NaCl, 5 mmol/L EDTA, 0.5 mmol/L Na_3VO_4, 50 mmol/L NaF, l0μg/ml aprotinin, 10μg /ml leupeptin, 1 0μg /ml pepstatin A, and l mmol/L phenylmethylsulphonyl fluoride. The cell extracts were used for immunoprecipitation reactions. The protein G immuno-precipitation kit from Sigma (St. Louis, USA) was used. The cell extracts were incubated overnight at 4℃, with P120ctn polyclonal antibody on protein G agarose beads. The beads were washed thoroughly after incubation and the sample was prepared for sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The blots were probed for E-cadherin protein. The amount of E-cadherin associated with P120ctn was calculated as a ratio of E-cadherin band optical density to P120ctn band optical density. The optical density analysis was carried out using LabWorks image analysis software (version 4.6; UVP-Media Cybernetics, Upland, USA).

Western blotting

Cell homogenates were boiled in SDS reducing bufer (5% beta-mercaptoethanol(v/v), 25% (v/v) glycerol, 2% (w/v) SDS, 0.01%(w/v) bromophenol blue, 0.0625 mol/L Tris-HCl, pH6.8)and resolved on 5%~15% discontinuous SDS-PAGE gels. Separated proteins were transferred to nitrocellulose membranes (Trans-BIot transfer, medium. BioRad, USA). Membranes were incubated in blocking buffer 5% nonfat dry milk in PBS, for 1 hour at room temperature. Mouse antiserum to P120ctn protein was diluted 1:5000 in 1% nonfat dry milk in PBS and incubated with the membrane overnight at 4℃. Membranes were washed 5 times with 0.1% Tween-20 in PBS. And subsequently incubated with a 1:2000 dilution of anti-mouse IgG conjugated to horseradish peroxidase (BioRad) for 1 hour at 37℃, immunolabeled bands were visualized by chemilumine scence (Amersham ECL kit) and exposed to film (Kodak BIOMAX ML.USA).

Protein structures and computational modeling

The amino acid sequence of human P120ctn(accession No. AF062344) was retrieved from the National Center for Biotechnology Information protein sequence database

(http://www.ncbi.nlm.nih.gov/). Computational modeling was carried out using the DeepView software Swiss PdbViewer. The P120ctn protein amino acid sequence was loaded into DeepView software and the 3-D structure was generated. Using the mutation feature in the software, variation of the amino acid at position 389 (Phe) was done in chain B of the protein. Chain A of the protein had the wild-type amino acid Phe at position 389. Visualization of the protein structure was carried out in the secondary structure form. Specific amino acid residues required for enzyme function were identified in the protein three-dimensional(3-D) model structure and amino acid distances were calculated using the distance measurement feature of the PdbViewer software. Using the amino-acid visualization tool in the software. Specific amino acids of interest were selected one at a time and distance measurements were carried out from amino acid 389. All the measurements from amino acid 389 were done from the side chain carbon atom. All distances were measured in angstrom units($\mathring{A}$) and are expressed here as absolute values and percentage variation.

Confocal microscopy

Cells were grown on a collagen—coated slide to confluence. They were fixed with formalin and washed with Tris-buffered saline. Double labeling was done using rabbit anti—P120ctn pAb (Transduction Biolabs, Bedford, USA)and rabbit anti-E-cadherin pAb (Upstate Biotechnology, Lake Placid, USA). Chicken anti-rabbit (Alexa Fluor 488; Molecular Probes, Carlsbad USA) labeled antibody was used as a secondary Preliminary computational modeling of P120ctn interactions with E-cadherin for P120ctn primary antibody, which showed green fluorescence。Goat anti-rabbit F(ab') 2 fragment (Alexa FIuor 633; Molecular Probes) labeled antibody was used as a secondary for E-cadherin primary antibody, which showed red fluorescence. Confocal microscopic observations were done using the LSM Meta 510 laser confocal microscope.

Statistical analysis

All data are expressed as mean±SE. Each cell line was treated as an individual sample. GraphPad Prism software (version 4.0; San Diego, USA) was used to fit the data and carry out the statistical analysis. A simultaneous comparison of groups was handled by one-way ANOVA. with Student-Newman-Keuls'post-hoc analysis. For analyzing the combination effects two-way ANOVA was used to test differences between the genotypes and various treatments. In all cases, significance was defined as $P<0.05$.

RESULTS

Identification of key amino acid residues

Using the National Center for Biotechnology Information sequence database and previous reports in the literature, key amino acids essential for P120ctn enzymatic activity were identified. These amino acid residues are listed in Table1, and were used for theoretical distance calculations in the computational modeling studies.

Table 1. Key amino acid residues required for P120 catenin function

Amino acid position	Structural significance	Function
Ser435--Tyr433	E-cadherin binding region	Regulation of protein
Arg398	Phosphorylation site	inducing angiogenesis and promoting nuclear translocation
Glu312	TCF/LEF transcription fact binding site	Modification of Rho-GTPaSe enzymatic function
Cys453	Kaiso binding region	Post-translational modification of conjunctional function and relieving transcriptional repression
Ser198	Palmitoylation	Targeting of the protein to the cell membrane and Golgi complex

Basal E-cadherin/P120ctn association is lower in the variant genotypes

Initially, immunoprecipitation studies were carried out and quantified as described in‘‘ Materials and Methods ”. The ratio of E-cadherin/P120ctn band optical density was 4.38±0.26 in Phe/Phe, but it was significantly lower in Phe/Leu (2.67±0.18) and Leu/Leu(1.38±0.31) variant genotypes (P<0.05) (Fig.1). These results show that under basal conditions the E-cadherin/P120ctn association is altered in the Leu variant genotypes.

Basal P120ctn membrane localization diminished in the Leu variant genotypes

We also carried out confocal microscopy under basal conditions an d probed for co-localization of P120ctn and E-cadherin. We observed greater co-localization of P120ctn and E-cadherin at the cell membrane in the wild-type cells. These studies showed that under basal conditions, the Phe/Phe wild-type cells have a greater P120ctn localization at the cadherinal regions on the cell membrane as compared to the Phe/Leu and Leu/Leu variant endothelial cells (Fig. 2).

Table 2. Predict amino acid distances (Å) in the P120catenin protein dimmer

	E-cadherin binding region		Phosphorylation site	TCF/LEF transcription factor binding site	Kaiso binding site	Palmitoylation
	Ser435	Tyr443	Arg398	Glu312	Cys453	Ser198
Phe 389	33.62	27.33	9.15	19.32	35.32	41.92
Leu389	30.49	23.86	8.31	17.87	33.87	39.87
Change	3.13	3.47	0.84	1.45	1.45	2.05
Change(%)	9.31	12.69	9.18	7.51	4.11	4.89

The percentage difference was calculated after variation of site 389. The percentage deviation was calculated from wild type amino acid Phe at position 389.

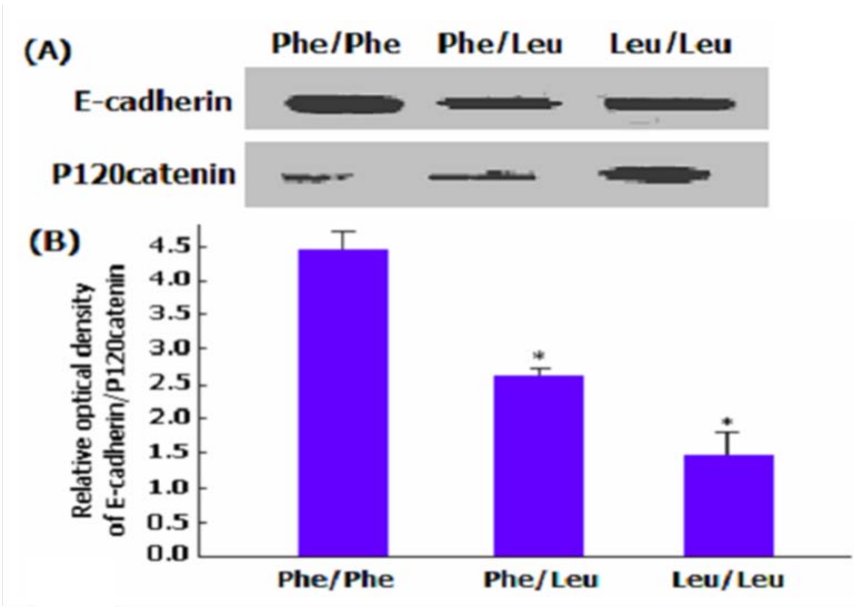

Figure 1. Diminished E-cadherin and P120catenin(E-cadherin/P120catenin) association in Leu variant endothelial cells under basal conditions

(A) E-cadherin/P120catenin association was investigated by immunoprecipitation (B) The E-cadherin/P120catenin association is expressed as a ratio of band optical density (OD). These data are represented as mean±SE. The E-cadherin/P120catenin protein association was significantly lower in both Phe/Leu and Leu/Leu variants compared with wild-type cells (*P<0.05)

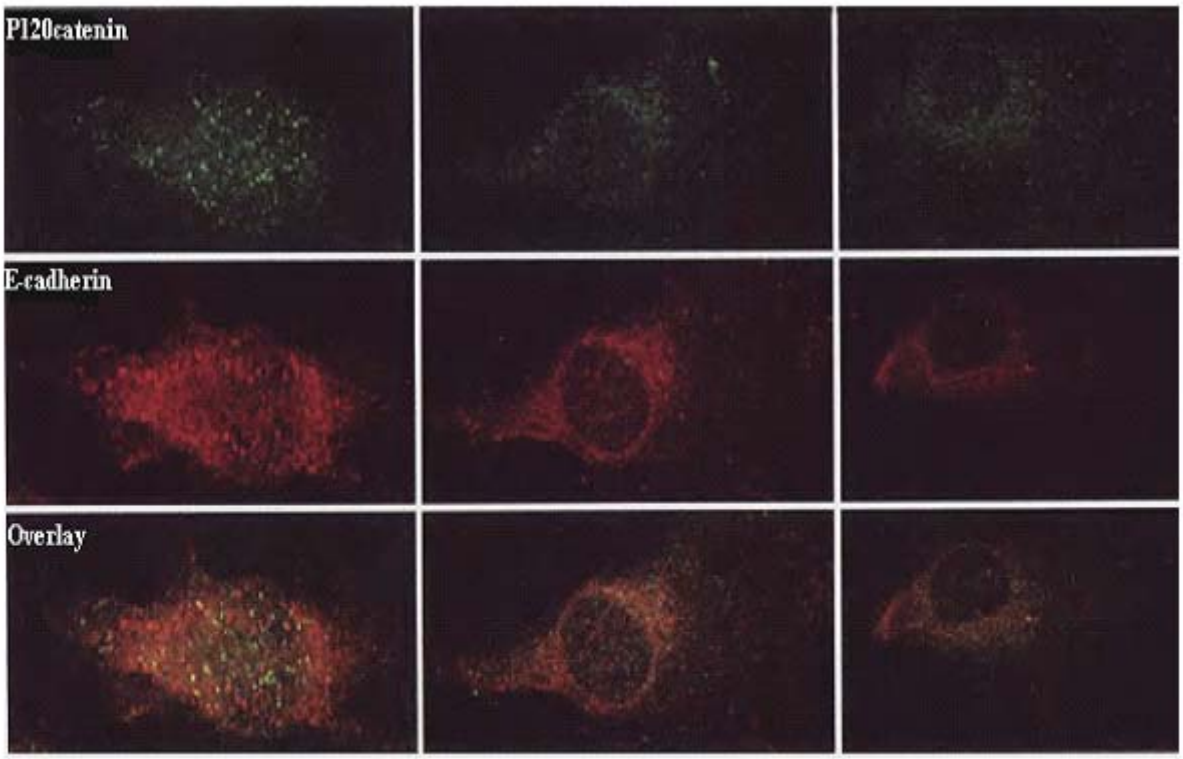

Figure 2. Altered P120catenin and E-cadherin co-localization by confocal microscopy in endothelial cells under basal conditions

wild-type and variant cells were probed for P120atenin and E-cadherin co-localization. The labeled P120catenin is represented as green fluorescence and E-cadherin as red fluorescence. The overlay (yellow) represents the co-localization of these two proteins. These data indicate that P120catenin and E-cadherin co-localization is diminished in variant genotypes under basal conditions.

Altered protein structural geometry in the variant amino acid chain

The 3-D protein structure was constructed using DeepView Software. The important substrate and co-factor binding sites were highlighted and shown in Fig. 3. Using the software, the amino acid at position 389 in chain B was mutated from Phe to Leu. In dividual amino acid distances from various substrate and co-factor binding sites were calculated for chain A (Phe at position 389) and chain B (Leu at position 389) based on this 3-D structure (Fig. 4). The absolute distances are shown in Table 2 and represented as angstrom units ($\mathring{A}$) as well as the percentage difference. Variation of the amino acid residue at position 389 from Phe to Leu in chain B resulted in altered protein geometry. The distance between the important sites and altered residues were consistently lower in chain B compared to chain A. The percentage differences were greatest in the E-cadherin binding region when amino acid variation occurred at position 389.

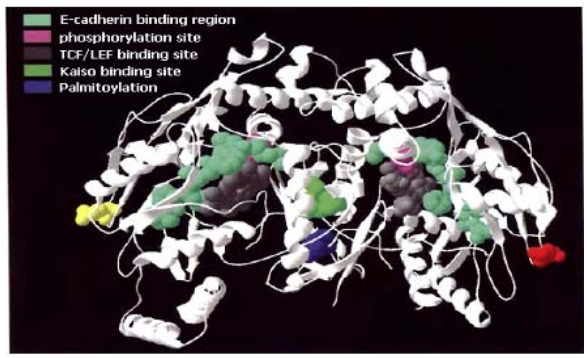

Figure 3. Theoretical 3-D structure of P120catenin with all the important sites for function

The P120catenin protein dimer is shown here with all the important sites highlighted. Chain A has Phe position 389 (yellow) and chain B has Leu at position 389 (red).

DISCUSSION

Theoretical protein modeling serves as an important tool for structure elucidation and prediction of protein interactions and structural features. Since the identification of the catenin family, many structural features have been

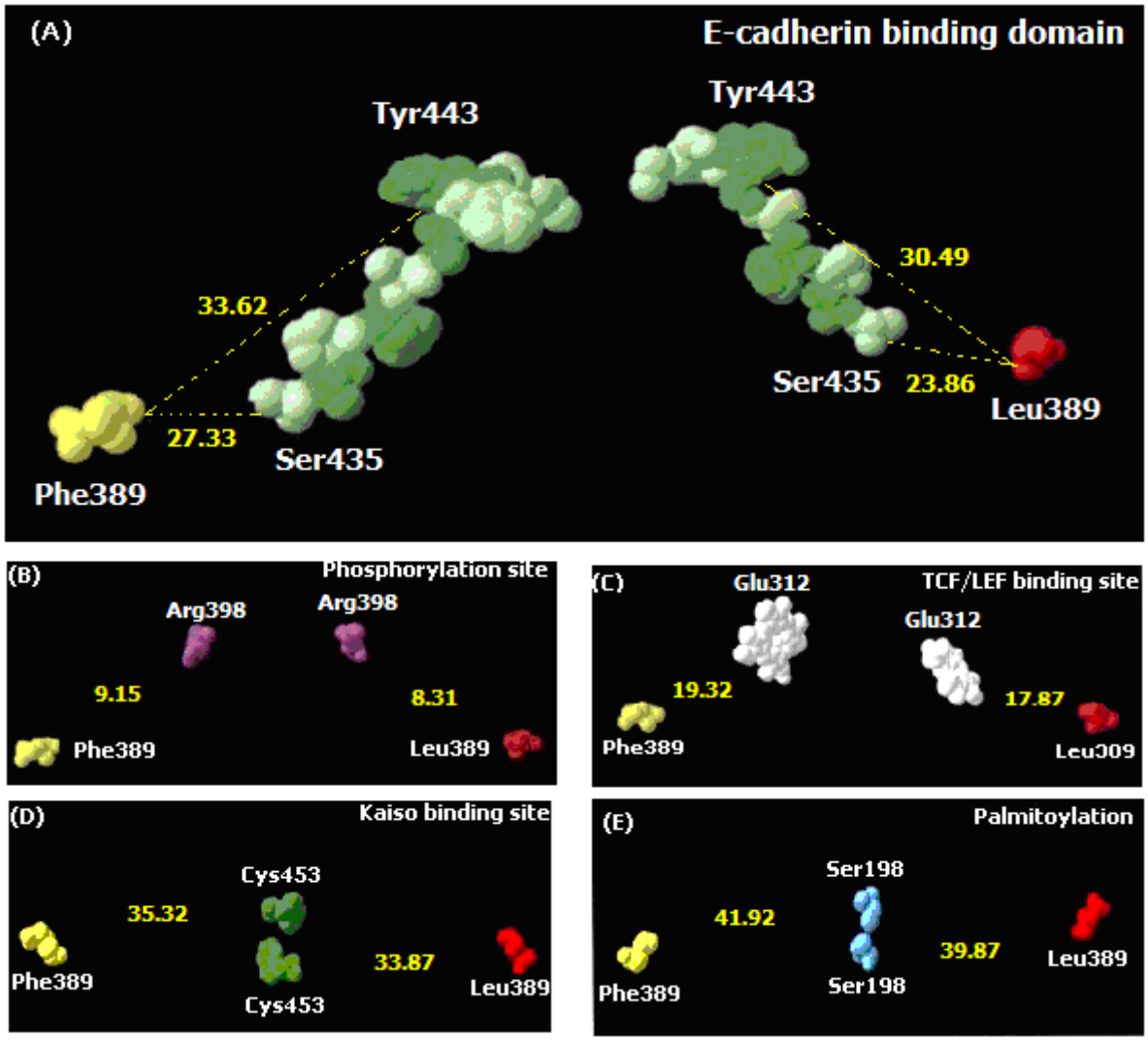

Figure 4. Altered the oretical amino acid distances in the variant P120catenin protein dimer Swiss-PdbViewer was used for theoretical distance calculations (yellow numbers) The amino acid at position 389 was mutated in chain B of the protein distances of the amino acid at position 389 were calculated from all the important sites for P120catenin protein function. (A) E-cadherin binding region on the P120catenin protein spans a region between amino acids Ser 435 and Tyr 443. The distance from amino acid 389 was measured to the start and the end of this E-cadherin binding region. (B) Arg392 is the Phosphorylation site. (C) Glu437 is the TCF/LEF transcription factor binding site. (D) Cys453 is the Kaiso binding site (E) The distances from Palmitoylation were measured. All distances are expressed i n Å

elucidated. Based on previous reports we compiled a list of the important sites that are necessary for P120ctn protein function (3,9,18). P120ctn is regulated in a complex manner and requires many post-translational modifications for proper functioning. Palmitoylation at the N-terminal are important for proper localization and targeting of the protein to organelles in the cell (4). The function of P120ctn is also dependent on Kaiso and TCF/LEF transcription factor availability as well as their interactions with other known regulatory proteins (E-cadherin and cdcv42) (5,16).

Given the importance of tight regulation of P120ctn protein and its important interactions with E-cadherin protein. We carried out P120ctn immunoprecipitation in endothelial cells that were previously genotyped for the presence of Phe389Leu polymorphism. We observed that in the Phe/Leu and Leu/Leu variant genotypes, the amount of P120ctn associated with E-cadherin was significantly lower. These observations were recapitulated by the confocal microscopic observations. Moreover, using a membrane enrichment method we also showed that the amount of P120ctn present in the membrane is significantly lower in the variant genotypes.

These data indicate that P120ctn is not localized properly to the membrane and its interaction with E-cadherin protein is altered in the Phe/Leu and Leu/Leu variants.

P120ctn is regulated in a cyclic manner and under static conditions, and it is bound to membrane through its interactions with E-cadherin (1,3). On stimulation, this interaction is dissociated and P120ctn enters the cytosol where it interacts with other proteins and co-factors to modulate RhoGTPase. Thus, E-cadherin bound P120ctn represents the available pool of P120ctn that can readily modulate RhoGTPase in the cell.

The 3-D protein structures might provide valuable insights into the molecular basis of protein function. Combining sequence information with the 3-D structure might give detailed information regarding the structural relevance of variations and protein function. Traditional methods like X-ray crystallography and nuclear magnetic resonance spectroscopy are time-consuming and require a pure form of the protein. We used the Swiss-Prot DeepView software to carry out theoretical structural modeling of the P120ctn protein. After resolving the structure and marking key sites on the protein, we observed that the amino acid at position 389 is not close to the active site pocket of P120ctn. The important site for P120ctn function is the dimerization site that has all the substrate and co-factor binding regions. Thus it is unlikely that the presence of the mutated amino acid (Leu) at position 389 will alter the function of P120ctn. We carried out computational modeling studies of the protein. Theoretical distance calculations revealed that variation of the amino acid at position 389 might affect protein geometry. The variation of an amino acid in one chain or both chains might introduce a structural variation in the protein structure and also affect the interactions of the protein. It is unlikely that this change in protein amino acid distances will affect the enzymatic pocket of the protein, but it might alter the protein localization and trafficking. It is not completely understood whether an amino acid distance change of 3-10 $\mathring{A}$ will actually make a difference in protein structure, but some reports suggest that a change of distance by 1 $\mathring{A}$ might affect the electrostatic energies to a great extent. We do not know the electrostatic energies in this modeling study, but it would be interesting to study these in light of the variation at position 389. Although this is not the most suitable method to characterize protein structural alterations, it does provide a good "starting point". The differences observed by using this method provide some insights into the structural and functional implications of a gene SNP. These observations provide preliminary evidence that the changes in protein structure might affect its interactions with substrate or co-factor.

In conclusion, we have shown, using a theoretical computational model, that variation of an amino acid at position 389 might affect the protein-protein interactions and localization of the P120ctn protein. These alterations might also affect the protein function and might explain the enhanced disease risk associated with the presence of Phe389Leu polymorphism in the P120ctn protein.

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