Author’s response to reviews

Title: Missense mutations in Desmocollin-2 N-terminus, associated with Arrhythmogenic right ventricular cardiomyopathy, affect intracellular localization of desmocollin-2 in vitro

Authors:

Giorgia Beffagna (giorgia.beffagna@unipd.it)
Marzia De Bortoli (marzia.debortoli@unipd.it)
Andrea Nava (andrea.nava@unipd.it)
Michela Salamon (mik@cribi.unipd.it)
Alessandra Lorenzon (alessandra.lorenzon@unipd.it)
Manuela Zacco (manuela.zacco@unipd.it)
Luisa Mancuso (luisa_mancuso@yahoo.it)
Luca Sigalotti (lsigalotti@cro.it)
Barbara Bause (barbara.bause@unipd.it)
Gianluca Occhi (gianluca.occhi@unipd.it)
Cristina Basso (cristina.basso@unipd.it)
Gerolamo Lanfranchi (lanfra@cribi.unipd.it)
Jeffrey A Towbin (jtowbin@bcm.tmc.edu)
Gaetano Thiene (gaetano.thiene@unipd.it)
Gian Antonio Danieli (danieli@bio.unipd.it)
Alessandra Rampazzo (alessandra.rampazzo@unipd.it)

Version: 3 Date: 18 September 2007

Author’s response to reviews: see over
Dear Dr. Alexandersson,

Enclosed you will find the revised version of our paper entitled “Missense mutations in Desmocollin-2 N-terminus, associated with Arrhythmogenic right ventricular cardiomyopathy, affect intracellular localization of desmocollin-2 in vitro”, which has been modified accordingly to Reviewers’ suggestions.

Enclosed you will find detailed answers to all the criticisms.

Hoping that the revised version fits with your expectations and is now suitable for publication in the *BMC Medical Genetics*, I look forward to hearing from you.

Yours sincerely,

Alessandra Rampazzo

---

*Answers to the Reviewer’s Comments*

Our response to the comments by the reviewers is given point-by-point below.

**Reviewer:** Ferhaan Ahmad

**Minor Essential Revisions**

1. I believe that the wildtype sequence at nucleotide 1034 is T, so that the codon is ATT encoding isoleucine (I), whereas the mutation at position 1034 is C, so that the codon is mutated to ACT encoding threonine (T). If this is correct, the mutation should be designated 1034T>C rather than 1034C>T throughout the manuscript.
We thank the Reviewer for picking up this mistake. The mutation has been correctly designated 1034T>C throughout the manuscript. We apologize for any confusion caused by our mistake.

2. To my eye, it appears that the p.I345T protein, like the p.E102K protein, is present to some extent at the membrane. The text implies that the p.I345T protein, unlike the p.E102K protein, is almost absent from the membrane. I am not certain that there is a real difference between the two mutant proteins. In fact, p.I345T does appear to co-localize somewhat with desmoglein, albeit less than wildtype desmocollin-2.

In neonatal rat cardiomyocytes, protein carrying the p.E102K mutation appeared evenly distributed (in dots) both in the membrane and in the cytoplasm (Figure 2, panel B), whereas protein carrying p.I345T mutation was predominantly localised in the cytoplasm (Figure 2, panel C), with a reduced membrane localisation not restricted to cell-cell junctions.

According to the Reviewer suggestion, we reported the same distribution of mutated protein observed in neonatal rat cardiomyocytes (Figure 2, panel E and F) in HL-1 cells.

The results section was changed as follow (page 12, line 14):
“p.E102K and p.I345T mutant fusion proteins showed the same distribution observed in neonatal rat cardiomyocytes (Figure 2, panel E and F).”

3. It is mentioned in the text that proband II-2, family #149, has left ventricular dilation. This fact is not mentioned in Table 2.

We thank the Reviewer for picking up the error in Table 2, that was modified.

4. Table 2 indicates that the 15-year-old daughter in family #170 has right ventricular abnormalities, but the text indicates that she is fully asymptomatic. To a non-clinical readership, “asymptomatic” may imply completely normal. It may be advisable to reword this sentence.

We modified the sentence and erased the term “asymptomatic” that could be misleading. We specified that the girl did not show any arrhythmic symptom, whereas the 2D-echocardiogram revealed mild right ventricular abnormalities.

The results section was changed as follow (page 11, line 13):
“The 15 years-old daughter, who did not show any arrhythmic symptom, was found to carry the same DSC2 mutation. The 2D-echocardiogram revealed mild right ventricular abnormalities, whereas 12-lead ECG was normal.”

5. Page 9, line 4. Please add “HL-1” in front of “cells.”

The text has been changed according to the Reviewer suggestion.

6. Reference 23 is incorrect. This paper appeared in J Cell Biol.

We have revised reference 23, according to the Reviewer suggestion.

7. Figure 2 legend. Please add “and HL-1 cells” after “two cardiomyocytes.”

Figure 2 legend has been revised, according to the Reviewer suggestion.
Discretionary Revisions

1. How were probands screened for mutations in DSP, PKP, DSG2, and TGFB3?

Probands were screened for mutations in DSP, PKP2, DSG2, and TGFB3 by denaturing high-performance liquid chromatography (DHPLC) and direct sequencing.

2. Do the authors feel that the E102K mutation, located in the propeptide region, alters cleavage? This residue is not in the cleavage consensus sequence. The authors may wish to speculate on the mechanism of pathogenesis of this mutation, given that it is not in the mature peptide.

The N-terminal pro-sequence is proteolytically cleaved off in the late Golgi and the mature cadherin is then transported to the plasma membrane. Proteolytic removal of the prosequence results in structural rearrangements within EC1 domain with the activation of adhesive properties. Mutation p.E102K, which is located in the propeptide region, 30 amino acids upstream the cleavage consensus sequence and alters a conserved amino acid, could affect the correct proteolytic cleavage which is of critical importance for DSC2 adhesive activity. To confidently make this statement, a western blot could be performed to look at the size of the mutant and WT proteins.

3. Table 2. RV size / function “M” and “m” presumably refer to major and minor diagnostic criteria. Please clarify.

As the Reviewer correctly stated, the term “M” and “m” was used referring to Major and minor criteria published in 1994. This information was added in the Table.

Reviewer: Luisa Mestroni

Discretionary Revisions

Only one clarification requested: I would be interested to know what the rationale was for using the two cell lines for their transfection experiments. Other than that I found the methods clearly explained, and the paper very interesting.

HL-1 cell line show spontaneous contractile activity and express many cardiac-specific genes typical of a differentiated adult cardiac phenotype. The HL-1 cells retain a pattern of gene expression characteristic of normal adult mouse myocytes. They express genes coding for adult protein isoforms (α-MHC and α cardiac actin), despite the fact that they are actively dividing. HL-1 cells therefore are unique in their ability to proliferate without reverting to an embryonic phenotype (Clycomb et al., 1998). These proprieties make HL-1 cells particularly attractive for studies that require expression of adult-specific genes.

On the other hand, the most used experimental models in cardiac research are the isolated whole heart and cultured cardiac cells. The neonatal rat cardiomyocytes model permits the study of many
morphological, biochemical and electrophysiological characteristics of the heart (Chlopcikova et al., 2001).

Because the same transfection results were obtained by using the two cell lines, we are confident that the detected missense mutations affect intracellular localization of desmocollin-2 in vitro.

Reviewer: Zahurul A. Bhuiyan

1) Mutation p.E102K: Only the proband fulfills the criteria for ARVC. But, what about the other carriers (father and two brothers), do they have any phenotype pertaining to ARVC? Authors might say that this mutation is less penetrant, but, which to my understanding will not be fully agreeable due to the numbers of unaffected. How is the clinical penetrance of DSC2 mutation carriers described by other authors compared to your findings?

2) I have similar question regarding the second mutation p.I345T.

We agree with this point raised by the Reviewer. This disorder is recognized as showing both low penetrance and variable expression. However, penetrance of the ARVC phenotype in families with disease-gene mutations is still poorly defined.

The two brothers of proband of family #149, aged 22 and 19 years old, carrying the p.E102K mutation, had features of ARVC, but did not achieve an adequate number of criteria to establish the diagnosis, whereas the father is completely asymptomatic. At the moment, we cannot exclude that the two young brothers could later show clinical signs of the disease. The same observations could be done in relation to family #170, in which the young daughter of the proband (aged 15 years old) resulted to carry the mutation but showed minor sign of the disease. Thus, the low penetrance of the DSC2 mutations could be age-related.

Unfortunately, it is not possible to ascertain the clinical penetrance of DSC2 mutation carriers described by other authors, since most of the family members of the probands were not available for clinical evaluation and/or genetic testing. However, the incomplete penetrance of ARVC has already been unequivocally demonstrated by the presence of healthy family members carrying mutations in the other disease genes (RYR2, DSP, PKP2 and DSG2).

We have addressed this point in the revised discussion (page 15, line 6):

“DSC2 mutations were detected in two ARVC probands and in four family members who met only minor diagnostic criteria. This is consistent with incomplete penetrance of the disease, as previously reported in ARVC patients family members carrying DSP, PKP2 and DSG2 mutations [7, 27-29]. However, due to the young age of most family members carrying the DSC2 mutations, we cannot exclude that some of them could later show clinical signs of the disease.”

3) Trafficking analysis: Authors have described that mutation causes halted trafficking. This should be clearly explained. I am sure authors have noticed that after transfection, cells transfected with wild type constructs show membrane localization, at the same time there are some cells which still have abundant expression in the cytoplasm. This does not mean that this is an intracellular protein, proteins are still not fully trafficked to the membrane.

My question:
Do you see GFP signal ONLY in the cytoplasm in case of both mutants?? This question applies to both p.E102K and p.I345T.
If it is completely intracellular, what will be your explanation about the healthy non-penetrant carriers? Is it not an important component in the ARVC pathogenesis?

As reported in figure 2, HL-1 cells transfected with wild type constructs show predominant membrane localization with a lower cytoplasmic GFP signal; however, proteins carrying p.E102K and p.I345T mutations are predominantly localised in the cytoplasm (Figure 2, panel E’’ and F’’), with a reduced membrane localisation not restricted to cell-cell junctions. Thus, in case of both mutants, GFP signal is not restricted to the cytoplasm and it is not completely intracellular.

We have addressed this point in the revised discussion (page 14, line 14):

“As previously reported, the wild-type DSC2a-GFP fusion protein was efficiently incorporated into desmosomes and did not exert dominant-negative effect when overexpressed [25]. A lower amount of GFP signal was detected in the cytoplasm, since proteins were still not fully trafficked to the membrane. Unlike wild-type DSC2, the N-terminal mutants were predominantly located in the cytoplasm.”