Somatic mutations in cardiac malformations

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It has come to our attention that there may be some confusion about the relationship of the data in two of our *NKX2-5* papers. Therefore, this correspondence aims to address the following issues: firstly, the relationship and the differences between our two *NKX2-5* papers^{1,2} and secondly, the research need for congenital heart disease (CHD) on the basis of somatic mutations.

The confusion about the relationship between the two publications may have originated because both papers are based on the analysis of 68 specimens of malformed hearts that had been conserved in formalin in Leipzig, and both papers report 35 nonsynonymous mutations in *NKX2-5* in the diseased but not in the healthy part of the heart. Our oversight of not citing the *Am J Pathol* (AJP) paper¹ in the *J Med Genet* (JMG) paper² may also have contributed to this confusion. Although it was not our intention to avoid citing the AJP paper in our JMG short report, different timelines in the submission and revision process of both papers prior to acceptance resulted in this oversight. We deeply regret this. It is, however, of considerable importance that in further publications on *NKX2-5* or submitted manuscripts on other cardiac specific transcription factors of the Leipzig collection of malformed hearts, both the AJP and JMG papers cited together, or the pertinent paper in appropriate situations.³⁻⁶ It is of equal importance that workers in the field cite both papers together.⁷

Herein, we wish to discuss the differences between both papers in their focal emphasis, to prevent ongoing confusion. Specifically, the sequence information of the 35 nonsynonymous mutations is reported in both papers, but there is a simple rationale behind this. In the JMG paper, we reported on the multiple haplotypes associated with the 35 nonsynonymous mutations and gave broad examples from our patient cohort. Note that for a given sequence variation, several configurations exist; for example, different clones carry either reference or variant alleles and this information is impossible to communi-

cate without naming the sequence in question. The information provided in the AJP and JMG papers is therefore different. In addition, in the JMG paper we reported that analysed clones both alleles were affected and provided information on 42 different clones associated with the pathology of seven individual cases. This was completely novel information and enabled us to put forward a hypothesis for multiple haplotypes as a result of a mixed population of cardiomyocytes carrying different mutations or de novo chromosomal rearrangements and gene duplications in the heart tissues of patients affected by mutations. This was the main emphasis of our JMG short report. Notably, we reported in JMG the full spectrum and combination of nonsynonymous mutations in each of the 67/68 patients.

Further points of consideration: The AJP paper is the source of information on:

- The detailed morphological characterisation of the Leipzig collection of hearts with complex malformations (not included in the JMG short report).
- Detailed methods on DNA isolation, detection, and amplification of *NKX2-5* fragments from formalin fixed hearts that had been stored for more than 40 years (not included in the JMG short report).
- Comprehensive spectrum of *NKX2-5* mutations (coding, untranslated, and intronic regions) detected in the malformed hearts (the coding (synonymous), untranslated and intronic regions were not reported in the JMG short report; in other words, we reported 18 additional mutations in the AJP paper).
- Clustering by response analysis of nonsynonymous *NKX2-5* mutations, and this analysis found five highly predictive mutations, such as Lys183Glu (third helix, homeo-domain), which was detected in 7/16 atrial septal defects and 22/23 atrioventricular septal defects, but not in 29 ventricular septal defects. This analysis also found that

Lys183Glu is highly associated with Down syndrome. Our functional study of *NKX2-5* mutations using the yeast transcriptional machinery demonstrates that Lys183Glu is a loss of function mutation, as recently reported by us.⁴

In strong contrast, the JMG short report:

- Put forward the hypothesis on the role of somatic mutations in cardiac malformations, particularly in multiple nonsynonymous *NKX2-5* mutations and multiple haplotypes based on sequencing of 42 different clones (not reported in AJP).
- Showed (table 2) the type, number, and combination of multiple, nonsynonymous mutations detected in individual hearts/patients within septal defect groups (not reported in AJP).
- Showed (table 3) detailed analysis of multiple haplotypes involving nonsynonymous mutations in several patients (not reported in AJP).

The molecular mechanisms leading to CHD are complex and the causes for the cardiac malformations observed in humans are still unclear. The exact causes of malformations in CHD are not known, although genetic and/or environmental factors as well as gene-environment interactions are possible culprits, resulting in mutations, chromosomal aberrations, or abnormal gene expressions.⁸ The classic view of organogenesis of the heart is also changing, in which the myocardium originates from two sources of myocardial cells rather than a single source as previously assumed.⁹ Inevitably, this new knowledge will have an impact in the understanding and prognosis of CHD.

Germline mutations in transcription factor genes such as *NKX2-5*, *TBX5*, and *GATA4* have been detected in patients with CHD.¹⁰⁻¹⁶ Recently, *NKX2-6*¹⁷ and *MYH6*¹⁸ have also been implicated in CHD. Mutations so far identified are mostly familial cases and families have unique ("private") mutations. Indeed, CHD can be sporadic, and many cases of CHD come from unaffected family members. A population based study investigated the genotypes of five known germline *NKX2-5* mutations in 227 babies with CHD, but did not detect any of these mutations.¹⁹

Previous genetic studies on CHD are based on blood samples and such analysis may not reveal complete genetic alterations leading to the disease, as has been shown in autoimmune lymphoproliferative syndrome, a disease of the immune system.²⁰ This study

demonstrates that somatic mutations can cause an autoimmune disease and that cells within the site of defect should be examined for genetic alterations. Although somatic mutations are well known in cancer, there is now evidence that such mutations can also cause disease in non-malignant settings.²¹ Somatic mutations can arise in discrete cell lineages early in embryonic development or during postnatal life, and their implications for disease depend on the stage they arise. Somatic mutations arising early during embryogenesis may affect the structure, function, and survival of tissues derived from that cell lineage.

To elucidate genetic alterations in the malformed heart itself, we (J Borlak's laboratory) started in 2002 the genetic analysis of cardiac specific transcription factor genes from the Leipzig collection of malformed hearts. Surprisingly, sequence analysis has been revealing mutations in diseased heart tissues, which are mainly absent in normal heart tissues of the same CHD patients. Even more unexpected are the frequent appearance of multiple mutations and different haplotypes. Together, these results suggest a somatic origin for mutations and hypermutagenicity in the diseased cardiac tissues of patients with CHD, the mechanism of which is unknown. The detection of multiple mutations and haplotypes may be explained by a mixed population of cardiomyocytes carrying different mutations or de novo chromosomal rearrangements and gene duplications. However, the frequency and the occurrence of multiple mutations (haplotypes) suggest novel mechanisms of genetic change such as gene conversion from a pseudogene. We analysed the functional significance of individual and multiple somatic *NKX2-5* mutants in CHD, particularly VSD and AVSD, using a yeast system.⁴ Our results suggest that different mutant haplotypes may lead to various disease phenotypes.

The use of archived DNA must be viewed with caution.²² We believe, however, that the multiple mutations we observed are unlikely to be artefactual. We confirmed mutations by double strand sequencing, PCR restriction fragment length polymorphism technique when possible, or cloning and resequencing of clones to separate variant alleles. Our assay shows that for *NKX2-5*, as well as for other analysed cardiac specific transcription factor genes, experiments involving independent PCR studies using the same DNA isolation batch within the diseased tissue, but different *Taq* polymerases give the same results, and independent PCRs

using different DNA isolation batches within the diseased tissue give basically the same variants, but may shift in mutation spectrum to detect additional variants, which is in agreement with the concept of somatic mutations and mosaicism. Many of the mutations were detected not just in single, but multiple cases. In the same malformed hearts, other genes are essentially unaffected, for example, cardiogenic *MEF2C*, *HEY2*, and *CFC1* or non-cardiogenic *HMGN4* and *BRCC2* in analysed fragments.⁶ We also studied the effects of formalin fixation on explanted hearts from mice and once again did not find sequence alterations, using our laboratory protocol.⁶

MOTIVATION IN STUDYING THE LEIPZIG HEART COLLECTION OF MALFORMED HEARTS

The Leipzig malformed hearts were collected over many years and the collection is a rare genetic resource in understanding congenital heart disease, because it is impossible to obtain such material from surgery. The heart collection gives us the opportunity to study the genetic changes in the whole explanted heart, rather than very minute biopsy material, which, if available, is often contaminated with DNA from unaffected tissue or the surrogate lymphocytic DNA. Thus, we diligently worked with the malformed hearts, and anyone who has worked with formalin fixed materials is very well aware of the difficulties and the high financial cost required to give confirmation of results, including independent PCR, cloning, and resequencing. Nevertheless, patients and the scientific community benefit from such studies in enabling new insights for molecular causes of CHD.

We hope that this correspondence will clarify the issue.

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