Title: mRNA expression patterns in human myocardial tissue, pericardial fluid and blood, and its contribution to the diagnosis of cause of death

Authors: Lucas González-Herrera[1], Ana Belén Márquez-Ruiz[1], María José Serrano[2], Valentín Ramos[3], José Antonio Lorente[1] and Aurora Valenzuela[1]

[1] Department of Forensic Medicine, Faculty of Medicine, University of Granada, Avenida de la Investigación 11, 18016 Granada, Spain.

[2] GENYO. Centre for Genomics and Oncological Research, Pfizer / University of Granada / Andalusian Regional Government, Avenida de la Ilustración 114, 18016 Granada, Spain.

[3] Forensic Pathology Service, Legal Medicine Institute of Malaga, C./ Fiscal Luís Portero García 6, 29010 Málaga, Spain.

Corresponding author:
Lucas González-Herrera
Email: lgh@ugr.es

Abstract

Gene expression has become an interesting research area in forensic pathology to investigate the process of death at the molecular level. The aims of this study were to analyze changes in gene expression patterns in relation to the cause of death, and to propose new molecular markers of myocardial ischemia of potential use for the postmortem diagnosis of early ischemic heart damage in cases of sudden cardiac death (SCD). We determined mRNA levels of five proteins related with ischemic myocardial damage and repair – TNNI3, MYL3, TGFB1, MMP9 and VEGFA – in specific sites of the myocardium, blood and pericardial fluid in samples from 30 cadavers with different causes of death (SCD, multiple trauma, mechanical asphyxia, and other natural deaths). TNNI3 expression in blood, and MMP9 expression in pericardial fluid, were significantly higher when the cause of death was mechanical asphyxia, probably because of the more sensitive response of these proteins to acute systemic hypoxia/ischemia. Specifically, among SCD cases, increased MYL3, VEGFA and MMP9 values in the anterior wall of the right ventricle were found when the confirmed cause of death was acute myocardial infarction (AMI). Higher TGFB1 expression was found in the interventricular septum when AMI was not the cause of death, most likely as a reflection of the short duration of ischemia. Molecular biology techniques can provide complementary tools for the forensic diagnosis of early ischemic myocardial damage and AMI, and may make it possible to determine the duration and severity of myocardial ischemia.

Keywords Postmortem mRNA expression – Cause of death – Sudden cardiac death – Myocardial ischemia – Forensic pathology

1. Introduction

Determining the cause of death can be difficult in forensic practice, especially in cases of sudden death. In adults, sudden death is defined as natural unexpected death occurring within 1 hour of the beginning of symptoms. Most cases of sudden death are related with cardiac disease, especially ischemic heart disease [1], with myocardial infarction (MI) being a common cause of sudden cardiac death (SCD). However, the postmortem

diagnosis of early ischemic lesions in the heart is a challenging problem in practical forensic medicine, since the typical morphological changes that occur in myocardial tissue because of ischemia require a minimum survival time to be detectable after death. Thus, it is often difficult to verify acute ischemic heart disease on autopsy. This makes it necessary to develop diagnostic tests that can ensure the accurate diagnosis of acute cardiac ischemia as the cause of SCD.

Many different approaches have been tried to find reliable markers of early myocardial ischemic damage. Morphological, histochemical, immunohistochemical and biochemical methods have all been tried for the diagnosis of myocardial injury, especially to diagnose early MI [2-10]. The use of conventional histological methods is limited because myocardial damage is usually manifested at least 4–6 hours after MI occurs [3, 11, 12]. In contrast, myocardial injury can be detected earlier with immunohistochemical techniques, i.e. about 1 or 2 hours after the beginning of ischemia, although these techniques are difficult to interpret due to artefacts caused by autolysis, and are not easy to use in routine forensic practice [3, 11, 13]. Biochemical methods include those based on the study of cardiac structural molecules such as cardiac troponin I and T in blood and pericardial fluid as postmortem markers of myocardial damage. In this case, however, it should be recalled that elevated levels of troponin in blood and pericardial fluid are also found in other causes of death such as hyperthermia, methamphetamine abuse and carbon monoxide poisoning, and the postmortem interval may also affect the detectability of these molecules. In addition, it is important to note that variability has been reported in the levels of cardiac markers in blood from different body locations in the same individual [2, 9, 11, 14, 15]. Thus, some authors have used different biochemical methods for cardiac tissues, such as the potassium/sodium ratio and formazan test [5, 6, 16]. Despite these limitations, biochemical methods have been shown to be more useful than histological methods in detecting myocardial damage at earlier stages [12, 17].

Aside from the approaches summarized above, postmortem gene expression studies are becoming an interesting field of research in forensic pathology to investigate the cause and process of death at the molecular level. In the context of postmortem diagnosis of the cause of death, gene expression studies in cardiac tissues and fluids from cadavers are needed to reach a better understanding of the underlying mechanisms of myocardial ischemia and its repair [10, 18], and to make it possible to identify early molecular markers of myocardial ischemia for the postmortem diagnosis of SCD.

In previous work we investigated the mRNA integrity, mRNA expression levels and postmortem stability of five genes related to ischemic myocardial injury and its repair – cardiac troponin I (*TNNI3*), myosin light chain 3 (*MYL3*), transforming growth factor beta 1 (*TGFB1*), matrix metalloprotease 9 (*MMP9*), and vascular endothelial growth factor A (*VEGFA*) – in postmortem samples from the heart (five myocardial sites) and body fluids (femoral vein blood and pericardial fluid). Our results showed that RNA extracted in all samples showed good integrity and remained stable for up to 24 hours after death [19].

The present study was designed to analyze changes in gene expression patterns in relation to the cause of death. The aim of this study was to identify differences in the expression levels of five proteins related with ischemic myocardial damage and repair (TNNI3, MYL3, TGFB1, MMP9, and VEGFA) in myocardial tissue, blood and pericardial fluid, in cadavers with different known causes of death. Our ultimate goal was to propose new molecular markers of myocardial ischemia of potential use for the postmortem diagnosis of early ischemic heart damage in cases of SCD.

2. Material and methods

2.1. Samples

Samples were obtained from a total of 30 cadavers (5 females and 25 males; mean age 65.03 ± 16.50 years; range 36–90 years) autopsied at the Institute of Forensic Medicine of Malaga (Spain) in accordance with the principles of the Declaration of Helsinki. The research protocol was approved by the Ethics Committee for Human Research of the University of Granada (Spain). All cadavers were kept at 4 °C until autopsy was performed at a known postmortem interval (mean PMI: 15.36 ± 5.67 hours, range 5–24 hours). Myocardial tissue, femoral vein blood and pericardial fluid were collected as described in a previous study [19].

The final cause of death was established on the basis of medical records, scene of death, autopsy, toxicological and histological findings. For this study the causes of death were grouped, based on pathophysiological similarities, into the following: (1) SCD, 14 cases (46.7%); (2) multiple trauma, 7 cases (23.3%); (3) mechanical asphyxia, 6 cases (20%); and (4) other natural deaths, 3 cases (10%) (Table 1).

Table 1 Case profiles

Cause of death	*n*	Male/Female	Age (years)		PMI (hours)	
			Range	Mean ± SD	Range	Mean ± SD
Sudden cardiac death	14	14/0	54–87	66.36 ± 10.45	5–23	13.68 ± 6.39
Multiple trauma	7	6/1	27–84	60.57 ± 25.80	10–22	17.22 ± 3.87
Mechanical asphyxia	6	2/4	36–90	64.50 ± 19.42	16–24	18.83 ± 2.84
Other natural deaths	3	3/0	55–79	70.33 ± 13.32	5–19	11.97 ± 7.08
Total	30	25/5	36–90	65.03 ± 16.51	5–24	15.36 ± 5.67

n, number of cases; SD, standard deviation; PMI, postmortem interval

Cardiopulmonary resuscitation treatment was not used in any individual included in the group of SCD. In this group, the pathological criteria for acute myocardial infarction (AMI) were confirmed in 5 cases (SCD Group 1), in which specific signs of myocardial coagulative necrosis with or without inflammatory cell infiltration were identified by hematoxylin–eosin histological examination of specific myocardial areas, as summarized in Table 2. In a second subgroup (SCD Group 2, n = 9) it was not possible to identify specific signs of myocardial coagulative necrosis, but a diagnosis of acute ischemic heart disease was established in 3 cases based on the presence of acute coronary artery thrombosis (Table 2). In the remaining 6 cases, chronic ischemic heart disease was demonstrated by the evidence of significant stenosis (>75%) in at least one of the main coronary arteries, with or without old myocardial infarction scarring (Table 2). The multiple trauma group included 2 individuals who were killed in motor vehicle collisions, 1 who died in a vehicle–pedestrian collision, 2 who fell from a height, 1 who feel at ground level, and 1 who fell on a stairway. Among individuals in the mechanical asphyxia group, 4 died from hanging and 2 from choking. The group of other natural deaths included 2 individuals who died of hypovolemic shock and 1 case of spontaneous subarachnoid hemorrhage. Morphological and histological analyses were performed as described previously [12, 17].

Table 2 Case profiles in sudden cardiac death (SCD) groups

	Age (years)	CVRF[a]	Microscopic examination (H&E)				Cause of SCD[f]
			Coronary arteries		Myocardial tissue		
			Atherosclerosis[b]	Stenosis[c]	AMI[d]	OMI[e]	
SCD	87		LAD/LCA/RCA	80/50/50	LVP/IVS		AMI
Group 1	65	CS	LAD/LCA/RCA	90/25/80	LVA	RVL	AMI
n = 5	70	EBP/ESTC/CS	LAD/LCA/RCA	75/25/75	LVL	LVAL	AMI
	65	EBP	LAD/LCA/RCA	40/80/50	LVA/IVS	LVA	AMI
	56	EBP	LAD/LCA/RCA	90/90/90	LVL/IVS	LVP	AMI
SCD	54	CS	LAD/LCA/RCA	80/15/100[AT]		RVL	IHD
Group 2	57	OMI	LAD/LCA/RCA	95/50/100[AT]		LVA&P	IHD
n = 9	62	CS	LAD/LCA/RCA	75/15/100[AT]		LVP	IHD
	59	DM/CS	LAD	80		LVP/IVS	CAD+OMI
	83	IC/DM/CS	LAD/LCA/RCA	30/75		LVA	CAD+OMI
	75	CS	LAD/LCA/RCA	90/90/90		IVS	CAD+OMI
	69		LAD/LCA/RCA	75/40/50		LVP/IVS/RVL	CAD+OMI
	54	CS/ESTC	LAD/LCA/RCA	50/25/90			CAD
	73	CS	LAD/LCA/RCA	75/25/40		LVP	CAD+OMI

n, number of cases

[a] CVRF, cardiovascular risk factors: CS, cigarette smoking; DM, diabetes mellitus; EBP, elevated blood pressure; ESTC, elevated serum total cholesterol; OMI, old myocardial infarction

[b] Atherosclerosis in coronary arteries: LAD, left anterior descending artery; LCA, left circumflex artery; RCA, right coronary artery

[c] Percentage degree of stenosis in each coronary artery as described in [b]. AT, acute coronary thrombosis in right coronary artery cases

[d] AMI, site of acute myocardial infarction: LVA, anterior left ventricle; LVP, posterior left ventricle; IVS, interventricular septum; RVL, lateral right ventricle

[e] OMI, site of old myocardial infarction scar: LVA, anterior left ventricle; LVP, posterior left ventricle; IVS, interventricular septum; RVL, lateral right ventricle

[f] AMI, acute myocardial infarction; IHD, ischemic heart disease; CAD, coronary arterial disease; OMI, old myocardial infarction

2.2. Expression studies

Gene expression was analyzed in human tissues and fluids as previously described [19]. Briefly, five specific sites in the myocardium were selected for molecular analyses: the anterior and posterior walls of the left ventricle, the interventricular septum, and the anterior and lateral walls of the right ventricle. Frozen tissue samples were disrupted and homogenized with TissueLyser LT (Qiagen, Hilden, Germany), and subsequently RNA was extracted with the RNeasy Fibrous Tissue Mini Kit (Qiagen). In pericardial fluid and blood samples, RNA was extracted with the PAXgene RNA Blood Kit (PreAnalityX, Hombrechtikon, Switzerland). The quantity and quality of RNA were determined by photometric analyses. RNA integrity was estimated with the RNA Integrity Number (RIN). Reverse transcription and real-time quantitative PCR (RT-qPCR) were performed with a two-step protocol. The QuantiTect Reverse Transcription kit (Qiagen) was used to synthesize complementary DNA (cDNA). Then mRNA expression levels were assayed with the QuantiTect SYBR Green PCR kit (Qiagen) and QuantiTect Primer Assay (Qiagen). For all samples, relative quantitative gene expression of *TNNI3*, *MYL3*, *MMP9*, *TGFB1* and *VEGFA* was analyzed. Glyceraldehide-3-phosphate dehydrogenase (GAPDH) was the housekeeping gene used for internal normalization. Relative quantitation was done by normalizing the Ct values of each sample gene to the Ct values of the housekeeping gene (ΔCt), and ΔCt was recorded as the difference between the Ct value of the gene of interest and the Ct value of the housekeeping gene. In addition, the mathematical model for relative quantification in RT-qPCR described by Pfaffl [20] was used.

2.3. Statistical analysis

Statistical analysis was done with SPSS version 23.0 software (IBM Corporation, Armonk, NY, USA). Descriptive statistics were recorded and expressed as the mean ± standard deviation of the mean. Statistical dependence between variables was determined with Spearman's rank correlation coefficient. The significance of the differences in expression levels between different causes of death was tested with the nonparametric Mann–Whitney U test for two independent samples, or the nonparametric Kruskal–Wallis test for more than one independent sample. A p value less than 0.05 was considered statistically significant.

3. Results

Postmortem mRNA levels of five proteins related with ischemic myocardial injury and repair (TNNI3, MYL3, TGFB1, MMP9 and VEGFA) were studied in myocardial tissue, blood and pericardial fluid from cadavers with different known causes of death (SCD, multiple trauma, mechanical asphyxia, and other natural deaths). When gene expression levels were compared across each of the four cause-of-death groups, significant differences in TNNI3 mRNA expression in blood samples were found between mechanical asphyxia and each of the other causes of death ($p < 0.05$, Fig. 1A). In pericardial fluid samples, significantly higher mRNA levels of MMP9 were found in the mechanical asphyxia group compared to the SCD group ($p < 0.05$, Fig. 1B). For the other genes, no differences were detected. Also, no significant differences in mRNA expression were seen between causes of death at any of the five sites of the myocardium analyzed.

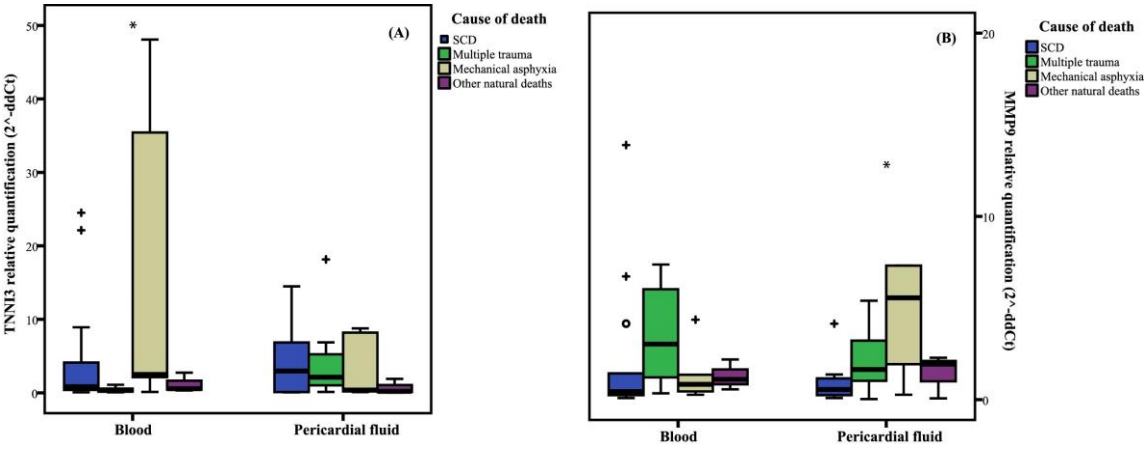

Fig. 1 Relative expression (2^{-ddCt}) of TNNI3 (A) and MMP9 (B) mRNA in blood and pericardial fluid in groups with different causes of death. The box represents the interquartile range, and the vertical lines represent the minimum and maximum values (adjacent values). Outliers are indicated by open circles, and are defined as values of more than 1.5 and less than 3 box lengths from the top end of the box. Extreme values are indicated by crosses, and are defined as values more than 3 box lengths from the top end of the box. (A) *Significant differences between mechanical asphyxia and each of

the other causes of death ($p < 0.05$). (B) *Significant differences between mechanical asphyxia and sudden ischemic cardiac death ($p < 0.05$).

To search for specific markers of early myocardial ischemia, the SCD group was divided for analysis into two subgroups as described above: SCD with a confirmed pathological diagnosis of AMI, and SCD without pathological evidence of AMI. The results showed that MYL3, VEGFA and MMP9 mRNA expression was significantly greater in the anterior wall of the right ventricle in individuals with confirmed AMI ($p < 0.05$, Fig. 2). The expression of TGFB1 mRNA in tissue from the interventricular septum also differed significantly between cases with AMI and without AMI, although at this site expression was higher in individuals with confirmed AMI ($p < 0.05$, Fig. 2). In contrast, no significant differences in mRNA expression were found between SCD subgroups when blood or pericardial fluid samples were compared.

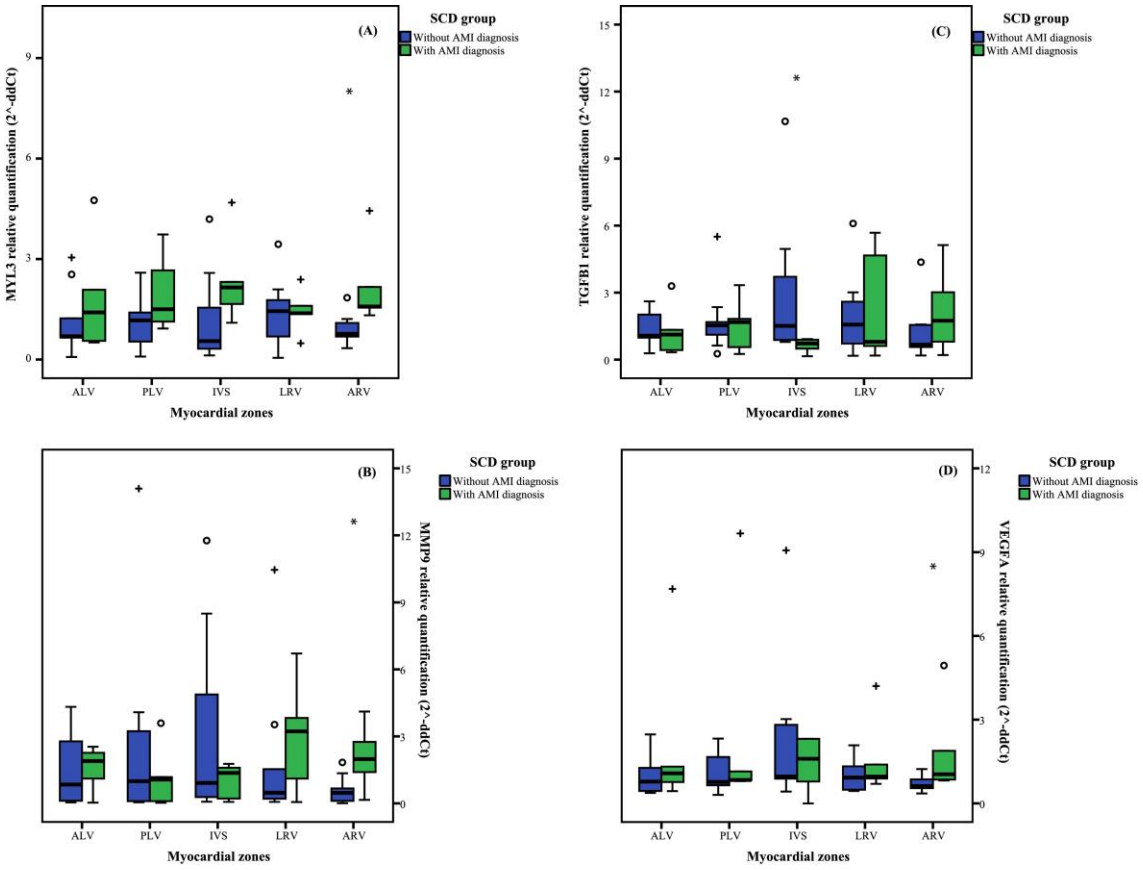

Fig. 2 Relative expression (2^{-ddCt}) of MYL3 (A), MMP9 (B), TGFB1 (C), and VEGFA (D) mRNA in five myocardial areas in the sudden cardiac death (SCD) group.

Myocardial area abbreviations: ALV, anterior left ventricle; PLV, posterior left ventricle; IVS, interventricular septum; LRV, lateral right ventricle; ARV, anterior right ventricle. Boxes and symbols are as explained in Fig. 1. *Significant differences between SCD cases with a confirmed diagnosis of acute myocardial infarction (AMI) and SCD cases without AMI ($p < 0.05$).

The correlations between mRNA levels for different genes in samples from the SCD group were significant and positive between VEGFA and MMP9 mRNA expression (rho = 0.736, $p < 0.01$) and between VEGFA and TGFB1 mRNA expression (rho = 0.719, $p < 0.01$) in the myocardium. Conversely, mRNA expression showed no significant relationships in body fluids (blood and pericardial fluid).

4. Discussion

There is no ideal method for the postmortem diagnosis of early myocardial ischemic injury, but mRNA expression profiling has become an interesting and potentially fruitful field of research in forensic pathology to investigate the cause and process of death at the molecular level. Studies of gene expression in different human tissues and fluids are important to elucidate the molecular mechanisms of pathological situations, because they offer insights into gene expression patterns in specific tissues at any given time. In fact, proposals have already been published for diagnostic methods based on changes in the RNA pool in response to the physiological needs of cells, tissues and organs; these methods are grounded on the variations in gene expression with certain pathologies and causes of death [21–24, 27–32]. Moreover, only a few studies have been done with forensic samples of myocardial tissue [13, 18, 27] or postmortem fluids [32].

Blood levels of the cardiac muscle structural protein TNNI3 are measured in daily clinical practice for the diagnosis of AMI. Another important structural protein in cardiac muscle is MYL3 [33-35], and the level of expression of both these structural proteins may change after ischemic cardiac disease. Another potentially informative protein for molecular studies is TGFB1, a cytokine that is strongly induced and rapidly activated in the infracted myocardium [29, 36, 37]. Similarly, MMP9 plays an important role in vessel matrix remodeling after ischemia, and contributes to left ventricle remodeling by altering the basement membrane, thus promoting molecular traffic

around myocardiocytes, vascular permeability, and left ventricle hydration [38-40]. Lastly, VEGFA is an angiogenic factor that is rapidly induced in the ischemic myocardium [41]. Therefore, information on the expression patterns of these structural, inflammatory and angiogenic molecules may help to identify early molecular markers of the myocardial response to cardiac ischemia, and thus aid in establishing the cause of death.

Our results showed that TNNI3 mRNA expression in blood was significantly higher in the mechanical asphyxia group than in the other causes of death compared here (Fig. 1). Given that TNNI3 is a specific structural protein of the heart, this finding implies that the myocardial injury produced by overall hypoxia and ischemia in fatal mechanical asphyxia is intense and even more marked than in SCD, in which hypoxia and ischemia are limited to a specific myocardial area. In samples of pericardial fluid, we also found higher values for MMP9 mRNA expression in the mechanical asphyxia group compared to the SCD group (Fig. 1), probably as a result of the much more sensitive response to acute systemic hypoxia/ischemia in the former. Therefore, mRNA levels of TNNI3 and MMP9 may depend on the intensity of hypoxic and ischemic stress in the myocardium during the process of death.

In the SCD group, increased MYL3, VEGFA and MMP9 mRNA expression in the anterior wall of the right ventricle were found in AMI cases, whereas higher TGFB1 mRNA expression was seen in the interventricular septum in SCD cases without AMI (Fig. 2). We note that the differences were significant only in certain parts of the myocardium; this result suggests that mRNA expression varies according to the site of the ischemic myocardial lesion. Particularly, lower TGFB1 mRNA expression in the interventricular septum in AMI cases appears to be related to the location of the AMI area. Our results are consistent with earlier reports of decreased TGFB expression rates in patients with AMI [29, 30], and significantly increased mRNA levels of VEGF in acute myocardial ischemia or evolving infarction – an indicator of the relatively prolonged duration of myocardial ischemia [13, 24, 25, 27].

These findings are directly related to the pathophysiology of myocardial ischemia. Thus, MMP9 and VEGFA are involved in a somewhat later response to ischemia; consequently their expression levels are higher in cases of SCD with confirmed AMI [24, 38-41]. Conversely, when AMI was not confirmed, and the individual died in a shorter time after the onset of symptoms, higher levels of TGFB1 expression were found, probably because this inflammatory protein is activated early in

the infracted myocardium [42] and can be induced by ischemic damage during relatively short survival periods. Finally, the fact that the expression level of the structural myocardial protein MYL3 was significantly higher in cases with AMI suggests that this protein is especially sensitive to myocardial necrosis. In addition, we found that the expression levels of VEGFA, MMP9, and TGFB1, molecules related to inflammation and angiogenesis, were correlated in myocardial tissue in response to myocardial ischemia, suggesting a coordinated response at the molecular level. The correlated expression of these proteins is evidence of a joint response to repair damaged heart tissue in myocardial ischemia – a combined response that was detectable even in postmortem tissue samples.

Our findings suggest that the duration of myocardial ischemia can be determined on the basis of mRNA expression profiles. For example, a profile in which TGFB1 expression predominates would indicate that acute death occurred in the context of a myocardial ischemic event in the acute phase (short duration). On the other hand, when MYL3, MMP9 and VEGFA expression predominates, death is likely to have occurred in the reparative phase of ischemia (substantial duration).

In deaths due to SCD, protein expression profiles can also help to distinguish between cases with AMI and those without confirmed AMI. This would be particularly useful in the latter group, in which the heart tissue is completely normal on autopsy. In these cases, we found higher levels of TGFB1 mRNA expression in the interventricular septum than in cases with confirmed AMI, in which the levels of MYL3, VEGFA and MMP9 mRNA were higher in the right ventricle anterior wall. These expression patterns provide evidence that allows us to identify cases of SCD without AMI.

In conclusion, by analyzing the expression of genes for molecules involved in cardiac structure, inflammation and angiogenesis, we were able to reconstruct the molecular response to myocardial ischemia, and at the same time to obtain evidence that can be used to diagnose the cause of death. Our results suggest that molecular biology can provide alternative tools for the forensic diagnosis of early ischemic myocardial damage and MI, and may eventually make it possible to determine the duration and severity of ischemic myocardial damage. Our results must be interpreted with caution because of our relatively small sample size, and should be complemented with studies of larger numbers of cases. This limitation notwithstanding, we conclude that gene expression analysis shows potential for use in order to determine the cause of death, and especially to differentiate between SCD and other causes.

Conflict of interest

The authors declare that they have no conflict of interest.

Funding

This work was supported by funding from the Centro para la Excelencia Forense en Andalucía (CEIFA-01/2008).

Acknowledgments

The authors gratefully acknowledge the scientific advice, guidance and support of Dr. Luis Javier Martinez from the Centro Pfizer – Universidad de Granada – Junta de Andalucía de Genómica e Investigación Oncológica (GENYO), and thank K. Shashok for improving the use of English in the manuscript.

References

1. De la Grandmaison GL. Is there progress in the autopsy diagnosis of sudden unexpected death in adults? Forensic Sci Int. 2006; 156:138-44.
2. Batalis NI, Marcus BJ, Papadea CN, Collins KA. The role of postmortem cardiac markers in the diagnosis of acute myocardial infarction. J Forensic Sci. 2010; 55:1088-91.
3. Campobasso CP, Dell'Erba AS, Addante A, Zotti F, Marzullo A, Colonna MF. Sudden cardiac death and myocardial ischemia indicators: A comparative study of four immunohistochemical markers. Am J Forensic Med Pathol. 2008; 29:154-61.
4. He K, Lu JP, Zhu XJ, Wang ZY. Research evolvement of postmortem diagnosis of early myocardial infarction in forensic pathology. Fa Yi Xue Za Zhi. 2006; 22:381-4.
5. Hougen HP, Valenzuela A, Lachica E, Villanueva E. Sudden cardiac death: A comparative study of morphological, histochemical and biochemical methods. Forensic Sci Int. 1992; 52:161-9.
6. Lachica E, Villanueva E, Luna A. Comparison of different techniques for the postmortem diagnosis of myocardial infarction. Forensic Sci Int. 1988; 38:21-6.

7. Mao RM, Zheng QQ, Li XL, Xiong CY, Zhu BL. The application of biochemical indexes detecting in sudden cardiac death in forensic autopsy. Fa Yi Xue Za Zhi. 2009; 25:451-4.
8. Tomaskova E, Vorel F. Some possibilities in the diagnosis of early acute ischaemic changes in the heart muscle in sudden death. Soud Lek. 2010; 55:32-5.
9. Zhu BL, Ishikawa T, Michiue T, Li DR, Zhao D, Kamikodai Y, et al. Postmortem cardiac troponin T levels in the blood and pericardial fluid. part 2: Analysis for application in the diagnosis of sudden cardiac death with regard to pathology. Leg Med (Tokyo). 2006; 8:94-101.
10. Sabatasso S, Moretti M, Mangin P, Fracasso T. Early markers of myocardial ischemia: From the experimental model to forensic pathology cases of sudden cardiac death. Int J Legal Med. 2018; 132:197-203.
11. Ortmann C, Pfeiffer H, Brinkmann B. A comparative study on the immunohistochemical detection of early myocardial damage. Int J Legal Med. 2000; 113:215-20.
12. Ramos V, Valenzuela A, Villanueva E, Miranda MT. Antioxidant-related enzymes in myocardial zones and human pericardial fluid in relation to the cause of death. Int J Legal Med. 1997; 110:1-4.
13. Zhu BL, Tanaka S, Ishikawa T, Zhao D, Li DR, Michiue T, et al. Forensic pathological investigation of myocardial hypoxia-inducible factor-1 alpha, erythropoietin and vascular endothelial growth factor in cardiac death. Leg Med (Tokyo). 2008; 10:11-9.
14. Osuna E, Perez-Carceles MD, Vieira DN, Luna A. Distribution of biochemical markers in biologic fluids: Application to the postmortem diagnosis of myocardial infarction. Am J Forensic Med Pathol. 1998; 19:123-8.
15. Zhu BL, Ishikawa T, Michiue T, Li DR, Zhao D, Oritani S, et al. Postmortem cardiac troponin T levels in the blood and pericardial fluid. part 1. analysis with special regard to traumatic causes of death. Leg Med (Tokyo). 2006; 8:86-93.
16. Lachica E, Villanueva E, Luna A. Regional study of free fatty acids and free carnitine behavior in cardiac tissue in relation to different causes of death. J Forensic Sci. 1988; 33:1483-90.

17. Gonzalez-Herrera L, Valenzuela A, Ramos V, Blazquez A, Villanueva E. Cardiac troponin T determination by a highly sensitive assay in postmortem serum and pericardial fluid. Forensic Sci Med Pathol. 2016; 12:181-8.
18. Son GH, Park SH, Kim Y, Kim JY, Kim JW, Chung S, et al. Postmortem mRNA expression patterns in left ventricular myocardial tissues and their implications for forensic diagnosis of sudden cardiac death. Mol Cells. 2014; 37:241-7.
19. Gonzalez-Herrera L, Valenzuela A, Marchal JA, Lorente JA, Villanueva E. Studies on RNA integrity and gene expression in human myocardial tissue, pericardial fluid and blood, and its postmortem stability. Forensic Sci Int. 2013; 232:218-28.
20. Pfaffl MW. A new mathematical model for relative quantification in real-time RT-PCR. Nucleic Acids Res. 2001; 29:e45.
21. Bauer M. RNA in forensic science. Forensic Sci Int Genet. 2007; 1:69-74.
22. Vennemann M, Koppelkamm A. mRNA profiling in forensic genetics I: Possibilities and limitations. Forensic Sci Int. 2010; 203:71-5.
23. Maeda H, Ishikawa T, Michiue T. Forensic molecular pathology: Its impacts on routine work, education and training. Leg Med (Tokyo). 2014; 16:61-9.
24. Lee SH, Wolf PL, Escudero R, Deutsch R, Jamieson SW, Thistlethwaite PA. Early expression of angiogenesis factors in acute myocardial ischemia and infarction. N Engl J Med. 2000; 342:626-33.
25. Xu X, Li J, Simons M, Li J, Laham RJ, Sellke FW. Expression of vascular endothelial growth factor and its receptors is increased, but microvascular relaxation is impaired in patients after acute myocardial ischemia. J Thorac Cardiovasc Surg. 2001; 121:735-42.
26. Zhao D, Zhu BL, Ishikawa T, Li DR, Michiue T, Maeda H. Quantitative RT-PCR assays of hypoxia-inducible factor-1alpha, erythropoietin and vascular endothelial growth factor mRNA transcripts in the kidneys with regard to the cause of death in medicolegal autopsy. Leg Med (Tokyo). 2006; 8:258-63.
27. Zhao D, Zhu BL, Ishikawa T, Quan L, Li DR, Maeda H. Real-time RT-PCR quantitative assays and postmortem degradation profiles of erythropoietin, vascular endothelial growth factor and hypoxia-inducible factor 1 alpha mRNA transcripts in forensic autopsy materials. Leg Med (Tokyo). 2006; 8:132-6.

28. Akatsu T, Nakamura M, Satoh M, Hiramori K. Increased mRNA expression of tumour necrosis factor-alpha and its converting enzyme in circulating leucocytes of patients with acute myocardial infarction. Clin Sci (Lond). 2003; 105:39-44.
29. Dabek J, Mazurek U, Gasior Z, Wilczok T, Kulach A, Kucia-Kuzma S. Transcriptional activity of genes encoding transforming growth factor beta and its receptors in peripheral blood mononuclear cells from patients with acute coronary syndromes. Int J Cardiol. 2006; 111:275-9.
30. Kempf K, Haltern G, Futh R, Herder C, Müller-Scholze S, Gülker H, et al. Increased TNF-alpha and decreased TGF-beta expression in peripheral blood leukocytes after acute myocardial infarction. Horm Metab Res. 2006; 38:346-51.
31. Zhu BL, Ishikawa T, Michiue T, Li DR, Zhao D, Tanaka S, et al. Postmortem pericardial natriuretic peptides as markers of cardiac function in medico-legal autopsies. Int J Legal Med. 2007; 121:28-35.
32. Chen JH, Inamori-Kawamoto O, Michiue T, Ikeda S, Ishikawa T, Maeda H. Cardiac biomarkers in blood, and pericardial and cerebrospinal fluids of forensic autopsy cases: A reassessment with special regard to postmortem interval. Leg Med (Tokyo). 2015; .
33. Barton PJ, Buckingham ME. The myosin alkali light chain proteins and their genes. Biochem J. 1985; 231:249-61.
34. Barton PJ, Robert B, Fiszman MY, Leader DP, Buckingham ME. The same myosin alkali light chain gene is expressed in adult cardiac atria and in fetal skeletal muscle. J Muscle Res Cell Motil. 1985; 6:461-75.
35. Zhang SZ, Xu Y, Xie HQ, Li XQ, Wei YQ, Yang ZM. The possible role of myosin light chain in myoblast proliferation. Biol Res. 2009; 42:121-32.
36. Bujak M, Frangogiannis NG. The role of TGF-beta signaling in myocardial infarction and cardiac remodeling. Cardiovasc Res. 2007; 74:184-95.
37. Frangogiannis NG. Regulation of the inflammatory response in cardiac repair. Circ Res. 2012; 110:159-73.
38. Lindsey ML. MMP induction and inhibition in myocardial infarction. Heart Fail Rev. 2004; 9:7-19.
39. Peterson JT, Li H, Dillon L, Bryant JW. Evolution of matrix metalloprotease and tissue inhibitor expression during heart failure progression in the infarcted rat. Cardiovasc Res. 2000; 46:307-15.

40. Vanhoutte D, Schellings M, Pinto Y, Heymans S. Relevance of matrix metalloproteinases and their inhibitors after myocardial infarction: A temporal and spatial window. Cardiovasc Res. 2006; 69:604-13.
41. Frangogiannis NG, Smith CW, Entman ML. The inflammatory response in myocardial infarction. Cardiovasc Res. 2002; 53:31-47.
42. Nian M, Lee P, Khaper N, Liu P. Inflammatory cytokines and postmyocardial infarction remodeling. Circ Res. 2004; 94:1543-53.