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Low myocardial transcript variant alt-a of cyclin dependent kinase inhibitor p21 expression differentiates hypothermia from cardiac/respiratory causes of death

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Abstract

Gene expressions in the myocardium have been shown to vary between different causes of death, which can be utilized in the recognition of varied processes. Our previous work with a limited number of cases showed a high messenger ribonucleic acid expression of the transcript variant alt-a of cyclin dependent kinase inhibitor p21 (*p21 alt-a*) in chronic cardiac ischemia deaths and a low expression in hypothermia deaths and acute myocardial ischemia deaths. In present work, *p21 alt-a* expression in the myocardium of human cadavers was calculated using glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) as reference gene. In this collection of 143 samples, the *p21 alt-a* expression was significantly lower in hypothermia than in chronic cardiac ischemic heart disease with ($P < .001$) or without ($P < .001$) acute myocardial infarction and in other cardiac and respiratory disease deaths ($P < .000$). Chronic ischemic heart disease in hypothermia cases did not increase the expression. The *p21 alt-a* expression did not correlate with postmortem interval, quality of RNA or with the age of the deceased. The *p21 alt-a* referenced to *GAPDH* expression in cadaver myocardium has apparent potential as a marker distinguishing between hypothermia and cardiac/respiratory diseases as causes of death.

Abbreviations: AHD = atherosclerotic heart disease, AMI = acute myocardial infarction, C + R = other cardiac and respiratory diseases, cDNA = complementary deoxyribonucleic acid, cH = hypothermia as a contributory cause of death, Cq = quantitative cycle in qPCR, GAPDH = glyceraldehyde-3-phosphate dehydrogenase, H = hypothermia as the primary cause of death, mRNA = messenger ribonucleic acid, *p21 alt-a* = the transcript variant alt-a of cyclin dependent kinase inhibitor p21, qPCR = quantitative polymerase chain reaction, RIS = RNA integrity score.

Keywords: cardiac/respiratory ischemia, cause of death, forensic, hypothermia, molecular marker

1. Introduction

Our research focuses on molecular mechanisms in hypothermia, but also on finding suitable molecular markers to be used in medico-legal death investigations. Hypothermia deaths are mostly associated with cold weather, but they occur also in temperate climate.[1] In cold climate countries, hypothermia victims are typically men who are found dead outdoors in winter, whereas in temperate climate countries the victims are predominantly elderly women with multiple illnesses.[2] Hypothermia is not always easy to confirm as a cause of death, and the investigation is mainly founded on the exclusion of other causes of death. In addition to the circumstances and environment, the urine adrenaline to noradrenaline ratio has been found to be valuable in the determination of possible antemortem stress.[3]

In 2016, for example, hypothermia mortality was 1.2 per 100,000 inhabitants in Finland,[4,5] which is consistent with 1.35 hypothermia deaths per 100,000 inhabitants in Northern Sweden.[6] Hypothermia deaths can easily be misclassified as cardiac deaths. Internationally, sudden cardiac deaths account for 15% to 20% of all deaths.[7] In Finland, chronic ischemic heart disease, also known as coronary artery disease and atherosclerotic heart disease, is the autopsy-verified cause of death in more than half of sudden cardiac deaths.[8] In 2016, the frequency of ischemic heart disease deaths was 186/100,000 in Finland, which is 19% of all deaths.[5]

Our goal is to find characteristic molecular markers for hypothermia to distinguish it from other causes of death, especially from deaths due to ischemic heart diseases. Cyclin-dependent kinase inhibitor p21 is a negative regulator of cell cycle progression and growth as well as an inhibitor of apoptosis.[9,10] In our previous

Editor: Robert Chen.

The authors have no conflicts of interest to disclose.

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How to cite this article: Kaija H, Pakanen L, Porvari K. Low myocardial p21 alt-a expression differentiates hypothermia from cardiac/respiratory causes of death. Medicine 2020;99:9(e19399).

Received: 6 November 2019 / Received in final form: 16 January 2020 / Accepted: 3 February 2020

http://dx.doi.org/10.1097/MD.0000000000019399

study, we quantified the messenger ribonucleic acid (mRNA) expression levels of *cyclin dependent kinase inhibitor p21* transcript variants *p21 V1*, *p21 alt-a*, and *p21* B.[11] In that preliminary study we identified pronounced mRNA expression of variant *p21 alt-a* in the myocardium to be characteristic for chronic ischemic heart disease deaths, and the expression of this variant was low in hypothermia. In that work, myocardial samples were analyzed from 3 cases per each cause of death.

In this study, we included cases with hypothermia as primary or a contributory cause of death, chronic ischemic heart disease with or without acute myocardial infarction (AMI), other heart diseases and chronic lower respiratory diseases. In this collection of 143 cases, the cardiac mRNA expression of *p21 alt-a* referenced to glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) proved to function as a practical marker distinguishing hypothermia from cardiac/respiratory diseases as causes of death.

2. Materials and method

2.1. Study material and permissions

Myocardial samples were collected in medico-legal autopsies in the Department of Forensic Medicine, University of Oulu, Finland and the National Institute for Health and Welfare, Oulu, Finland.

Fresh, nonformalin-fixed tissue samples from the anterior wall of the left ventricle were collected for research purposes. The samples were stored at −80°C.

The permissions to use cadaver tissue samples and data from medico-legal death investigations were granted by the National Supervisory Authority for Welfare and Health (Dnro 2932/06.01.03.01/2013) and the National Institute for Health and Welfare (Dnro THL/479/5.05.01/2013), respectively. The Northern Ostrobothnia Hospital research ethics committee approved the study protocol (31/2007).

2.2. Study groups

Based on causes of death in autopsy reports, 143 myocardial samples were randomly chosen among frozen tissues collected between 2000 and 2017 in the Northern Ostrobothnia District of Finland. The study consisted of cases of hypothermia (n = 30), chronic ischemic heart disease (n = 50), other forms of congestive heart diseases (n = 23), hypertensive heart disease with or without heart failure (n = 24), chronic lower respiratory diseases (n = 16). Postmortem intervals (time or estimated time from death to autopsy) were collected from autopsy reports.

Study groups and analyzed variables are presented in Table 1: Hypothermia deaths were divided into H: hypothermia as the primary cause of death (n = 23), hypothermia as a contributory cause of death (cH) (n = 7), and a combined group of H + cH (n = 30). The groups formed from the chronic ischemic heart disease deaths were: atherosclerotic heart disease (AHD) (n = 18), AHD + AMI: atherosclerotic heart disease with AMI (n = 32), and a combined group of AHD and AHD + AMI (n = 50). The combined hypothermia group was further divided into groups H + cH + AHD: hypothermia cases with contributing atherosclerotic heart disease (n = 13) and H + cH − AHD: hypothermia cases without contributing atherosclerotic heart diseases (n = 17). Other cardiac and respiratory diseases group (C + R), consisted of heart diseases and pulmonary diseases mostly with contributing chronic ischemic heart disease (n = 63). More detailed information of each group is presented in Table 2.

2.3. Extraction of total RNAs, RNA integrity measurements, and measurements of p21 alt-a and GAPDH mRNA

The total RNA was extracted from the myocardium with a miRNeasy kit (Qiagen, Hilden, Germany) using an automated QIAcube sample preparation instrument (Qiagen), and the quality of RNA (RNA integrity score) was analyzed using the QIAxcel method (Qiagen) according to the manufacturer's protocol. A High Capacity complementary deoxyribonucleic acid (cDNA) Reverse Transcription kit (AB Applied Biosystems, Foster City, CA) was used for the synthesis of cDNAs.

Quantitative polymerase chain reaction (qPCR) amplifications were made with a Rotor Gene Q (Qiagen) instrument using gene-specific primers (Sigma-Aldrich, Haverhill, UK) and Maxima SYBR Green qPCR Master Mix (Fermentas, Glen Burnie, MD, USA) for mRNAs as reported earlier.[11] Primers for *p21 alt-a* were: F: 5′-CTGTTTTCAGGCGCCATGT-3′ and R: 5′-GCCATTAGCG-

Table 1

Description of parameters for the study groups.

Causes of death	Abbreviation		Sex	Age, yr	RIS	PMI
		n	M/F	Median ± SEM	Mean ± SEM	Mean ± SEM
Hypothermia as primary cause	H	23	14/9	65.0 ± 3.9	5.2 ± 0.3	6.0 ± 1.2
Hypothermia as contributory cause	cH	7	6/1	78.0 ± 4.4	5.8 ± 0.2	5.0 ± 1.0
Combined hypothermia group	H + cH	30	20/10	68.0 ± 3.3	5.4 ± 0.3	5.8 ± 1.0
Atherosclerotic heart disease	AHD	18	16/2	63.0 ± 2.7††,§§§	3.9 ± 0.2$^{**\dagger\dagger\dagger\ \ddagger\ddagger\ddagger\ \S\S\S}$	4.4 ± 0.5
Atherosclerotic heart disease and acute myocardial infarction	AHD + AMI	32	26/6	64.0 ± 1.9†,§§	3.5 ± 0.2$^{***\dagger\dagger\dagger\ \ddagger\ddagger\ddagger\ \S\S\S}$	3.8 ± 0.2
Combined ischemia group	AHD and AHD + AMI	50	42/8	63.5 ± 1.6$^{\dagger\dagger,\ \S\S\S}$	3.7 ± 0.1***,†††,‡‡‡,§§§	4.0 ± 0.2
Hypothermia + chronic ischemia	H + cH + AHD	13	8/5	75.0 ± 2.4**,‡	5.7 ± 0.2	5.2 ± 0.6
Hypothermia − chronic ischemia	H + cH − AHD	17	12/5	55.0 ± 4.5†,§§	5.1 ± 0.4	6.4 ± 1.7
Other congestive heart diseases Hypertensive heart diseases Chronic lower respiratory diseases	C + R	63	53/10	66.0 ± 1.4§§	3.6 ± 0.2***,†††,‡‡‡,§§§	4.4 ± 0.2

AHD = atherosclerotic heart disease, AMI = acute myocardial infarction, cH = hypothermia as a contributory cause of death, PMI = post mortem interval, RIS = RNA integrity score.
$^{***}P < .001$, $^{**}P < .01$, $^{*}P < .05$ compared with hypothermia as primary cause of death (H).
$^{\dagger\dagger\dagger}P < .001$, $^{\dagger\dagger}P < .01$, $^{\dagger}P < .05$ compared with hypothermia as contributory cause of death (cH).
$^{\ddagger\ddagger\ddagger}P < .001$, $^{\ddagger\ddagger}P < .01$, $^{\ddagger}P < .05$ compared with combined hypothermia group (H + cH).
$^{\S\S\S}P < .001$, $^{\S\S}P < .01$, $^{\S}P < .05$ compared with hypothermia + chronic ischemia (H + cH + AHD).

Table 2

Frequencies of diagnoses as causes of death in different groups.

Causes of death	H n=23	cH n=7	AHD n=18	AHD + AMI n=32	C + R n=63
Hypothermia	23	3			
Other specified effects of reduced temperature		4			
Atherosclerotic heart disease	8	5	18	32	57
Myocardial infarction		2		32	31
Cardiomegaly	4	1			7
Cardiomyopathy					4
Non-rheumatic mitral/aortic valve disorders		1			14
Hypertensive heart disease	1	3			24
Emphysema					5
Chronic obstructive pulmonary disease		1			4
Asthma					7
Alcohol consumption	13	3	5	1	5
Alcohol intoxication	1	1			2
Other than circulatory or respiratory causes	9	4			10

AHD = atherosclerotic heart disease, AMI = myocardial infarction, cH = hypothermia as contributory cause of death, C + R = other heart and respiratory diseases, H = hypothermia as primary cause of death.

CATCACAGT-3′ and for *GAPDH*, F: 5′-TGGAAGGACTCAT-GACCACA-3′ and R: 5′-TTCAGGTCAGGGATGACCTT-3′.

The expression of *p21 alt-a* was calculated with the $2^{-\Delta\Delta Cq}$ method, where Cq is quantitative cycle in qPCR and $\Delta\Delta Cq = (Cq_{p21alt\text{-}a} - Cq_{GAPDH})_{sample} - (Cq_{p21alt\text{-}a} - Cq_{GAPDH})_{control}$.[12] cDNA synthetized from Commercial Human Heart Total RNA (Clontech/Takara, Mountain View, CA; catalog No. 636532, RNA pooled from 10 male/female Caucasians, age range 25–51, cause of death trauma) was used as control.

2.4. Statistical analysis

IBM SPSS statistics, version 24 (Armonk, NY), was used to perform the statistical analyses. The SPSS tests Shapiro–Wilk and Kolmogorov–Smirnov were used to test the normality of the data. Not all data followed normal distribution, and in addition, some of the groups were small. Therefore, the pair-wise comparisons between reasonable pairs of groups were made with the nonparametric Mann–Whitney test (statistical significance level set at $P < .05$). Correlations between variables were analyzed by the Pearson correlation test (2-tailed; statistical significance level set at 0.05).

3. Results

3.1. qPCR analyses

Average Cq values with standard errors and statistical differences between the groups are presented in Table 3. The Cq values of both *p21 alt-a* and *GAPDH* differed statistically between hypothermia and other groups. The *p21 alt-a* expressions in all hypothermia groups (H, cH and H + cH) were significantly lower compared to all the chronic ischemic heart disease groups (AHD, AHD + AMI and AHD & AHD + AMI), and compared to the group of C + R (Fig. 1). The *p21 alt-a* mRNA level was also lower in cases where hypothermia was the main or a contributory cause of death together with chronic ischemic heart disease (H + cH + AHD) compared to the chronic ischemic heart disease groups (AHD, AHD + AMI and AHD & AHD + AMI) (Fig. 1). There was no statistically significant difference between the

Table 3

Average Cq values with standard errors.

Causes of death	*p21 alt-a* mean ± SEM	*GAPDH* mean ± SEM
H	28.63 ± 0.28	17.42 ± 0.23
cH	29.10 ± 0.55	18.12 ± 0.45
AHD	29.91 ± 0.30**	20.62 ± 0.31***,†††
AHD + AMI	29.70 ± 0.21**	20.20 ± 0.28***,††
C + R	30.28 ± 0.20***	21.00 ± 0.19***,†††

AHD = atherosclerotic heart disease, AMI = myocardial infarction, cH = hypothermia as contributory cause of death, C + R = other heart and respiratory diseases, GAPDH = glyceraldehyde-3-phosphate dehydrogenase, H = hypothermia as primary cause of death, p21 alt-a = transcript variant alt-a of cyclin dependent kinase inhibitor p21, SEM = standard error of mean.

$^{***}P < .001$, $^{**}P < .01$ compared with H.

$^{\dagger\dagger\dagger}P < .001$, $^{\dagger\dagger}P < .01$, $^{\dagger}P < .05$ compared with cH.

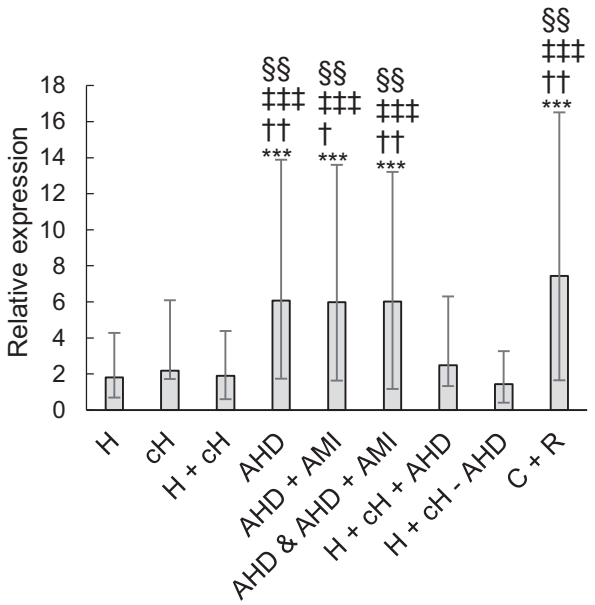

Figure 1. mRNA expression of *p21 alt-a* referenced to *GAPDH* in the myocardium of human cadavers. The causes of death in hypothermia were: H = hypothermia as the primary cause of death, cH = hypothermia as a contributory cause of death, and in chronic cardiac ischemia: AHD = atherosclerotic heart disease, AMI = acute myocardial infarction. Other causes of death were: C + R = other cardiac and respiratory diseases. Error bars represent 95% confidence intervals. $^{***}P < .001$ compared with H, $^{\dagger\dagger}P < .01$, $^{\dagger}P < .05$ compared with cH, $^{\ddagger\ddagger\ddagger}P < .001$ compared with H + cH, $^{\S\S}P < .01$ compared with H + cH + AHD.

hypothermia groups with (H + cH + AHD) or without chronic ischemia (H + cH − AHD) (Fig. 1). There was also no statistical difference between the chronic cardiac ischemic cases with (AHD + AMI) or without (AHD) AMI.

Alcohol consumption/intoxication was associated with 6 of the 50 chronic ischemic heart disease deaths (AHD and AHD + AMI) and with 18 of the 30 cases with hypothermia as main or contributory cause of death (H + cH). There were no significant differences in the *p21 alt-a* expressions between cases with an alcohol-related cause of death and those without such diagnosis within the groups.

3.2. RNA quality, age at death, and postmortem interval

The mean RNA integrity score values of cardiac samples were statistically higher in the hypothermia groups compared to groups without hypothermia (Table 1).

There were no correlations either between RNA integrity score and Cq values or between RNA integrity score and *p21 alt-a* transcript levels.

There were significant differences between the median ages of the groups (Table 1). However, there were no correlations between ages and *p21 alt-a* transcript levels.

Postmortem intervals varied between 3.8 ± 0.2 days (AHD + AMI) and 6.4 ± 1.7 days (H+cH-AHD) (Table 1), but there were no statistical differences between the groups and there were no correlations between post mortem intervals and *p21 alt-a* transcript levels.

4. Discussion

The postmortem diagnosis of hypothermia can be challenging, and heart diseases often pose a differential diagnosis. The determination of hypothermia as a cause of death is typically based on police investigations on the scene, knowledge about the finding place of the deceased and environmental circumstances, absence or presence of specific macroscopic and microscopic findings, medical history and the use of medication, drugs or alcohol. Postmortem biochemistry is increasingly used in medico-legal death investigations, and more recently also molecular pathology.[13] Our recent finding concerning microRNA U6B small nuclear RNA (RNU6B) expression[14] could be useful in forensic molecular diagnostics.

Based on our previous work,[11] the transcript variant *p21 alt-a* mRNA of the cyclin dependent kinase p21 was chosen as a potential candidate marker to distinguish between hypothermia and ischemic cardiac deaths. In the current large sample collection, the Cq values of the reference gene *GAPDH* also varied significantly between the groups. Therefore, the use of *GAPDH* as a reference in mRNA expression profiling is not optimal in current type of sample collection, if regulatory mechanisms are investigated. However, the expression level of *p21 alt-a* referenced to *GAPDH* proved to be an effective marker distinguishing between hypothermia and chronic ischemic heart disease and other cardiac/respiratory diseases. The *p21 alt-a* expression was lower in hypothermia cases including cases with both hypothermia and chronic ischemic heart disease together as causes of death. In contrary to our preliminary study with a limited number of samples, *p21 alt-a* expression levels were comparable between chronic ischemic heart disease deaths with or without AMI.

The quality of mRNA is variable in postmortem samples. The most precise and widely used way for sample quality evaluation is the ribosomal-based method of RNA categorizing.[15] Based on higher RNA integrity score values, the quality of RNA was better in hypothermia deaths than in the other groups in this study. However, the RNA integrity score values did not correlate with *p21 alt-a* expression. This is in line with the finding that short amplicons are practically independent of the quality of RNA in the applied qPCR method.[16] This explains also the lack of correlation between transcript levels and post mortem intervals.

Differentiating between hypothermia and chronic ischemic heart disease as the main cause of death is often problematic in practical medico-legal work. The finding that the expression level of *p21 alt-a* referenced to *GAPDH* is significantly lower in hypothermia deaths, even with contributing ischemic heart disease, than in deaths caused by ischemic or other heart/respiratory diseases can provide an additional tool for postmortem diagnostics.

The strength of this work is comprehensive study material, but the expression level of *p21 alt-a* referenced to *GAPDH* is limited to diagnostic purposes and does not clarify exact cellular regulatory mechanisms of *p21* gene.

In summary, a low cardiac expression level of *p21 alt-a* referenced to *GAPDH* is characteristic of hypothermia deaths even with concomitant chronic ischemic heart disease.

Acknowledgments

The authors thank Mirja Mäkeläinen and other laboratory staff of the Faculty of Medicine, University of Oulu, Finland.

Author contributions

Conceived and designed the work: Helena Kaija, Katja Porvari.
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[5] Official Statistics of Finland (OSF): Causes of Death [e-publication]. ISSN=1799-5078. 2016. Appendix Table 2a. Deaths by Underlying Cause of Death and by Age in 2016, Both Sexes. Helsinki: Statistics Finland. Available at: http://www.stat.fi/til/ksyyt/2016/ksyyt_2016_2017-12-29_tau_002_en.html. [Accessed November 9, 2018]
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