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Brief Communication

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

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Abstract

Recent advances in molecular biology have suggested the potential usefulness of mRNA analyses in postmortem investigations of fatal mechanisms. The aim of the present study was to establish quantitative assays of oxygen-regulated factors including erythropoietin (EPO), vascular endothelial growth factor (VEGF) and hypoxia-inducible factor 1 alpha (HIF1A) mRNAs, and to investigate the postmortem stability of these mRNA transcripts in forensic autopsy materials. Relative quantification of EPO, VEGF and HIF1A mRNAs, based on the TaqMan reverse transcription-polymerase chain reaction (RT-PCR), was performed on autopsy tissue specimens from the heart ($n=10$), brain ($n=10$), kidney ($n=16$) and lung ($n=8$) after preservation at room temperature for various storage times. VEGF and HIF1A mRNA gradually degraded in patterns similar to glyceraldehydes-3-phosphate dehydrogenase (GAPDH) mRNA used as an endogenous reference. Accordingly, the relative quantification of VEGF/GAPDH and HIF1A/GAPDH changed little up to 48 h postmortem in tissue samples from the brain, kidney and lung except for a mild deviation of HIF1A in the myocardium. However, the status was different for EPO mRNA, with extraordinary stability for postmortem degradation and a marked postmortem time-dependent increase in the EPO/GAPDH ratio for all tissue samples. The present study suggested the potential for applying quantitative analyses of mRNA transcripts to autopsy materials and indicated the significance of investigating degradation profiles prior to carrying out relative quantification of target mRNAs in autopsy materials.
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Keywords: Erythropoietin; Vascular endothelial growth factor; Hypoxia-inducible factor 1 alpha; Postmortem degradation; Messenger ribonucleic acid

1. Introduction

Oxygen homeostasis undergoes a sharp deterioration during fatal processes, especially in asphyxia-related death. Recent advances in molecular biology suggest that hypoxia-inducible factor 1 (HIF1), a transcription factor that functions as a global regulator of hypoxic gene expression, plays a major role in the response of hypoxia together with other factors including erythropoietin (EPO) and vascular endothelial growth factor (VEGF) [1,2]. The expression of these factors involved in oxygen homeostasis increase at the protein and messenger ribonucleic acid (mRNA) levels in organisms or cells that suffer from hypoxia or ischemia [3,4]. Therefore, it may be possible to investigate fatal processes involving advanced hypoxia by evaluating the mRNA levels of the above-mentioned oxygen-regulated factors in autopsy materials based on Taqman RT-PCR. For this purpose, the postmortem influence on the yield and quality of total RNA extraction should be considered for autopsy materials. Previous animal experiments showed that postmortem mRNA degradation profiles of some house-keeping genes were similar, and relative quantification of induced target mRNA interleukin-1β could be detected for at least 3 days postmortem, although the degradation rate of the target mRNA was higher [5]. For human materials, quantitative RT-PCR assay was reported to have no relationship with postmortem interval up to 3 days in

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the lung [6]. However, postmortem stabilities of HIF1A, EPO and VEGF mRNAs have not been investigated. Various factors involved in postmortem interference including sampling site, postmortem environments and period should be carefully considered before evaluation with regard to the cause of death.

In the present study, we established quantitative assays of EPO, VEGF and HIF1A mRNA transcripts in the present study, and investigated their postmortem degradation profiles in various tissues from forensic autopsy materials.

2. Materials and methods

2.1. Subjects and samples

Tissue specimens from the heart (left and right ventricle, $n=10$), brain (cerebral cortex and midbrain, $n=10$), kidney (left and right cortex, $n=16$) and lung (left and right lobe, $n=8$) were collected at the given site, respectively, in selected witnessed forensic autopsy cases at our institute (ca. 24 h of postmortem interval). Systematic sampling was not available in some cases due to pathological findings or injuries to target organs. The seasons of death spread over about half a year (January–March, $n=8$; April–July, $n=8$). The causes of death were fatal injury ($n=4$), asphyxia ($n=4$), fire fatality ($n=2$), drowning ($n=2$) and myocardial infarction ($n=4$). Each tissue sample was divided into six portions (0.1 g each), preserved separately in sterilized covered 2.0 ml micro tubes (BIO-BIK) at room temperature (24 °C) for 0, 6, 12, 24, 48 and 72 h, then immediately submerged in 1 ml of RNA stabilization solution (RNAlater™ Ambion, Austin) and stored at 4 °C for less than 1 week until RNA extraction. Total RNA was isolated with ISOGEN (Nippon Gene, Toyama) according to the manufacturer's instructions, quantified by spectrophotometry, and stored at −80 °C until use.

2.2. Methodology of TaqMan RT-PCR and relative quantification of mRNA transcripts

Quantitative assays were based on TaqMan RT-PCR. The relative quantification of mRNA transcripts was carried out by the comparative C_T ($\Delta\Delta C_T$) method, the theoretic basis of which was previously described in detail [5,7]. The amount of target, normalized to an endogenous reference (glyceraldehydes-3-phosphate dehydrogenase, GAPDH), and relative to a calibrator, was determined by evaluating the expression $2^{-\Delta\Delta C_T}$, where $\Delta\Delta C_T = \Delta C_{T,s} - \Delta C_{T,cb}$, $\Delta C_{T,s}$ was the ΔC_T value of a sample, $\Delta C_{T,cb}$ that of the calibrator, ΔC_T the difference in threshold cycles between the target and reference, and C_T a numerical value given as parameters by ABI PRISM 7700 Sequence Detector software, having a negative correlation with the initial amount of mRNA in reaction system. TaqMan RT-PCR was performed on an ABI PRISM 7700 Sequence Detector (Perkin–Elmer Applied Biosystems, Foster City), and both kinds of TaqMan RT-PCR Core Reagents kits for RT-PCR reaction were purchased from Perkin–Elmer Applied Biosystems.

2.3. Oligonucleotide sequence and TaqMan RT-PCR conditions

The sequence of primers and probes for EPO, VEGF and GAPDH mRNA transcripts were quoted from Refs. [7–9], but that for HIF1A mRNA transcripts was designed with the use of Primer Express (version 1.0, Perkin–Elmer Applied Biosystems, Foster City) from the GenBank database (BT009776). The forward primer sequence was 5′-TTCCAGTTACGTTCCTTCGATCA-3′; the reverse primer sequence was 5′-GCTGGAATACTGTAACTGTGCTTTG-3′; and the probe sequence was 5′-CAGTTCCGCAAGCCCTGAAAGCG-3′. RT-PCR was performed in a 50 μl reaction system with the use of the TaqMan EZ RT-PCR kit and TaqMan Gold RT-PCR kit (Table 1). Negative controls were simultaneously performed.

2.4. Statistical analyses

The Wilcoxon Rank-Sum Test was used to evaluate the differences between groups with different storage times, and a P-value less than 0.05 was considered to be statistically significant.

Table 1
TaqMan RT-PCR conditions

Components	Final concentration	
	EZ RT-PCR kit	Gold RT-PCR kit
Rnase-free water	See below[a]	See below
TaqMan EZ buffer	1×	–[b]
TaqMan buffer A	–	1×
Mangnese acetate (mM)	3	–
Magnesium chloride (nM)	–	5.5
dA/C/GTP (μM)	300	300
DUTP (μM)	600	600
Forward/reverse primer (nM)	200	200
Probe (nM)	100	100
rTth DNA polymerase (U/μL)	0.1	–
AmpliTaq Gold DNA polymerase (U/μL)	–	0.025
AmpErase UNG (U/μL)	0.01	–
Multiscribe reverse transcriptase (U/μL)	–	0.25
Rnase inhibitor (U/μL)	–	0.4
Total RNA (μg)	0.5	0.5

Thermal cycler parameters of EPO and VEGF using the TaqMan EZ RT-PCR kit: 50 °C for 2 min, 60 °C for 30 min, and 95 °C for 5 min for 1 cycle; 94 °C for 20 s, 62 °C for 1 min for 40 cycles. Thermal cycler parameters of HIF1A and GAPDH using the TaqMan Gold RT-PCR kit: 48 °C for 40 min, and 95 °C for 10 min for 1 cycle; 95 °C for 15 s, 60 °C for 1 min for 40 cycles.

[a] Volume of Rnase-free water varies with that of total RNA.

[b] Component was not contained.

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