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Review

RNA in forensic science

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Abstract

RNA analysis offers insight into diseases and mechanisms leading to death and could develop into a valuable tool for diagnosis of the cause of death in forensic pathology. Other possible applications include the determination of the age of wounds and injuries and of the post-mortem interval. The molecular identification of body fluids by analysis of cell-specific mRNA expression already represents a new technique supplementing DNA analysis in forensic cases. This review explains and discusses principles, techniques and applications by offering a complete and comprehensive overview of research results in forensic RNA work.
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Keywords: RNA; Wound age determination; Real time PCR; Body fluid identification; Gene expression

Contents

1. Introduction 69
2. Forensic applications 70
   2.1. Post-mortem RNA stability 70
   2.2. Identification of body fluids 70
   2.3. Wound age determination 71
   2.4. Determination of post-mortem interval and RNA quality 71
   2.5. Determination of the age of stains 71
   2.6. Functional status of cells and organs 72
3. Summary 72
   References 73

1. Introduction

Molecular forensic science has been predominated by DNA research in the past decade. The reason for this disproportion is the overwhelming success of DNA technology in criminalistics which has revolutionized modern policing—another cause is the gap between this sophisticated technology and the still very traditional way of practicing forensic pathology in the autopsy theatre. Furthermore, RNA was and is notorious for its allegedly rapid post-mortem and in vitro decay which has kept forensic scientists from investing time and money into this field.

However, times are changing and the enormous potential of RNA technologies together with reports of unexpectedly high stability in certain conditions have stimulated forensic researchers all over the world to start exploring the RNA world. In forensic science, the first paper dealing with RNA was published in 1984 describing post-mortem RNA synthesis [1], the next one already addressing the question of gene expression analysis in post-mortem tissues not before 1994 [2]. In the following years, there were only few and scattered reports until the end of the century when the availability of RT-PCR stimulated scientists to explore the forensic potential of RNA for the identification of body fluids [3] and the investigation of molecular changes in wound healing [4]. In the last years, the number of forensic RNA papers has not risen dramatically but steadily and there seems to be a growing interest in the forensic

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Table 1

Detection of cell-specific gene expression
Identification of body fluids
Quantification of RNA degradation
Determination of post-mortem interval
Determination of the age of stains
Assessment of RNA quality
Reactive changes of gene expression
Wound age determination
Determination of vitality and survival time
Functional status of cells and organs
Diagnosis of cause and mechanism of death

community for this field of molecular biology. This review is aimed to provide the reader with an overview of the present knowledge and the most recent developments and to outline the possible and practical use of RNA techniques in forensic science (Table 1).

2. Forensic applications

2.1. Post-mortem RNA stability

After death RNA is degraded by ribonucleases already present in the cell and/or originating from bacteria or other environmental contamination. Physical and unspecific chemical factors add to this effect. It is well known that ribonuclease-rich organs such as pancreas and liver exhibit rapid RNA fragmentation [5,6] whereas other tissues such as brain show much greater stability up to 96 h post-mortem [7–13]. Prolonged post-mortem RNA stability has been described in fetal and neonatal lung tissue [14], human bone [15], retina [16] and blood [17,18], although the last two studies dealt with virus RNA. The first systematic studies in humans have confirmed this and shown that mRNA suitable for RT-PCR can be extracted after post-mortem intervals (PMI) of more than 96 h although there is an increasing fragmentation and reduction in overall RNA [13]. In rats, these results have been reproduced with the brain as most stable mRNA donor and the liver as poorest RNA source [19]. A systematic microarray study of post-mortem RNA degradation in adult mice has shown that a subgroup of mammalian mRNA transcripts carrying the AUUUA motif in the 3′ untranslated region (3′UTR) are particularly susceptible to PMI-related degradation showing a 2-fold reduction in RNA amount but that almost 90% of all mRNAs are more stable [20].

Apart from the overall stability of RNA in post-mortem tissue it is crucial to know whether correlations are present between the levels of mRNA transcripts within a single brain. Results are so far promising because it could be shown that the quantitative order of different mRNAs within the same brain is constant although mRNA levels between brains from different individuals vary considerably [21,22]. However, other authors are less optimistic and call for careful selection and testing of markers used [23].

Agonal factors such as coma and hypoxia are believed to affect RNA integrity in post-mortem human brain samples whereas the PMI is considered to be without significant influence by some authors [24–27]. However, the evaluation of agonal factors by arbitrarily established scores (e.g. subdivision of the duration of agony into fixed time intervals of <10 min, <1 h, <24 h and >24 h) seems to be inadequate from a forensic point of view and determination of PMI is not well documented in these studies which partially obtained materials from brain banks [28].

On the other side, this question directly affects a possible forensic application of mRNA analysis: if agonal factors really influence post-mortem mRNA integrity and/or pre-mortem gene expression, mRNA analysis could be used as diagnostic tool to assess the type and duration of agony and coma and the degree of hypoxia which would provide valuable information about the cause and time-course of death.

2.2. Identification of body fluids

DNA analysis allows the identification of individuals from tiny stains containing only a few nucleated cells but not determination of the type and origin of the evidence. In many situations this question could be of importance to establish a link between evidence and crime: blood stains attributed to rape or homicide by the police but claimed to be of menstrual origin by the suspect or the presence of saliva or vaginal secretions on penile swabs, just to mention a few examples. Easily available presumptive tests with high specificity exist for semen (PSA) but not for other body fluids. Cell-specific mRNA detection could fill this gap by offering crucial advantages: high sensitivity due to the possibility of PCR amplification, high specificity due to the pattern of gene expression unique for the functional status of cells and organs and simultaneous DNA isolation without loss of material, if necessary [29,30].

A number of studies have shown that mRNA suitable for RT-PCR can indeed be extracted from dried stains stored for up to 15 years [31]. However, it should be kept in mind that unfavourable storage conditions can affect RNA more than DNA and that small-sized samples, which provide results in DNA testing, may fail to contain sufficient RNA for analysis.

mRNA markers have been identified for the forensically most relevant body fluids based on functional differences of the cells and tissues involved. For menstrual blood matrix metalloproteinases, a zinc dependent enzyme family cleaving extracellular matrix proteins, have been shown to be highly expressed in menstruating endometrium and to allow the reliable differentiation from non-menstrual blood [32]. These results have been confirmed by other research groups [33–35]. In our experience, the identification of menstrual blood might become the most important body fluid test because blood stains are the most frequent type of evidence. However, the availability of markers for other substrates such as semen or saliva certainly will be extremely helpful in forensic casework [34,36,37]. A compilation of markers evaluated so far is given in Table 2. Juusola and Ballantyne have condensed several

Table 2
Compilation of molecular markers evaluated for the identification of body fluids by RT-PCR

Blood
β-Spectrin (SPTB) [34]
Porphobilinogen-Deaminase (PBGD) [34]
Hemoglobin alpha locus 1 (HBA) [37]
Menstrual blood
Matrix metalloproteinase 7 (MMP-7) [32,34]
Matrix metalloproteinase 11 (MMP-11) [32,35]
Saliva
Statherin (STATH) [34]
Histatin 3 (HTN3) [34]
Semen
Protamine 1 (PRM1) [34,36]
Protamine 2 (PRM2) [34,36]
Kallikrein 3 (KLK = PSA) [37]
Vaginal secretions
Human beta-defensin 1 (HBD-1) [34]
Mucin 4 (MUC4) [34,37]

markers in a multiplex PCR assay which offers a convenient way to screen for body fluids in only one reaction [34].

The most impending pitfall of this new technique is the problem of false negative results which has been addressed by quantifying PCR results and defining cut-off values for the control marker but cannot be totally avoided and limits in some respect the practical value of the technique because in many criminal cases it is the negative result which matters (although this is a side effect of every analysis technique dealing with unknown, small and potentially degraded samples).

2.3. Wound age determination

One of the most important questions to be answered in forensic pathology is the age of external or internal wounds. Four main areas are of interest: the order in which wounds have been inflicted, whether the wound was produced pre- or post-mortem, the survival time after infliction of a lethal injury and the question whether a given injury is related to an incident. Virtually all the recent investigations into this field are based on reactive molecular changes within the wound margins. Whereas the last decade of the 20th century was predominated by immuno-histochemical studies requiring the rather tedious counting of immuno-positive cells [38,39], a slight change of focus towards RNA-based methods can be seen during the last years. Initiating the synthesis of new proteins or the increase in concentration of proteins already present in lower amounts in the cell requires enhanced translation activity on the ribosome level which can be achieved by offering more mRNA to the ribosome. Thus, every change of the functional activity of a cell will include changes in gene expression; in other words, new mRNAs coding for proteins involved in the cell reaction to injury will be synthesized and the transcription rate of mRNAs coding for proteins already present in the cell but needed in higher amounts will be increased. This increase can be measured by the new quantitative methods mentioned above and it is beyond any doubt that this approach is promising and capable of constituting a new era in forensic pathology. The questions which remain to be answered are the evaluation of suitable markers and the adaptation of RNA technology to the special circumstances and needs of forensic casework. Up to now only animal tissue has been investigated for mRNA markers as indicator of wound age. Promising results have been obtained for basic fibroblast growth factor [40] which is peaking 48 h after injury, e.g. in cerebral wounds, tissue-type plasminogen activator peaking at 1 h post-injury [41] and interleukin 10 [4]. In further studies, pro-inflammatory cytokines have been investigated with the time-course of the increase in expression varying considerably between the markers [42].

Newer studies addressed immediate changes in gene expression after pressure to the neck by fluorescent differential display-PCR and found a number of genes responding to mechanical pressure within 30 min after death [43]. This could be used for diagnosis of compression of the neck and establishes a direct link to Section 2.5 because in this case mRNA analysis is directly related to the cause of death.

A different approach based on the capability of post-mortem cells to synthesize DNA and RNA after death has been suggested but obviously never was continued [1,44]

2.4. Determination of post-mortem interval and RNA quality

The idea of using nucleic acid degradation for the determination of the post-mortem interval has been raised by several authors [45]. The more rapid RNA decay would predispose it for the intermediate post-mortem interval which involves the period between the first 24 h and the onset of decomposition. As mentioned above, some non-forensic authors deny a close link between RNA degradation and the PMI. However, studies performed under forensic conditions have demonstrated that there is a clear correlation which has the potential to be used for this purpose [13] although realistically it has to be conceded that these data have been obtained under well controlled circumstances (constant ambient temperature) and that the non-systematic nature of RNA degradation might render it difficult or even impossible to come to consistent conclusions from degradation measurements in bodies from real scenes. However, these and other studies have shown that RNA remains largely intact for a considerable time period (up to 24–48 h) after death as long as bodies are stored at appropriate ambient conditions. Furthermore, assessing RNA degradation by RT-PCR is believed to produce more accurate results than traditional techniques such as comparing the ribosomal 18S and 28S bands [27]. Therefore, measuring RNA degradation can provide information about RNA quality in tissues which are used for research or for diagnostic purposes such as wound age determination.

2.5. Determination of the age of stains

Two studies have examined RNA degradation as parameter for the determination of the age of dried blood stains using

special techniques based on RT-PCR such as duplex RT-PCR, competitive RT-PCR and a combination of both by comparing mRNA to rRNA in a single reaction [31,46]. This subject would be of outmost importance because at present there is no method available to assess the age of stains. In principle RNA fragmentation continues in vitro even if the material is completely dehydrated. However, this process is slow and shows considerable variation. The only possible differentiation so far is between recent (<1 year) and old (>8 years) stains [31]. In the future, real time PCR or chip technology could offer a more sensitive approach allowing age determination within a range of up to 150 days [46] although the main problem, the unpredictable conditions forensic stains are exposed to will persist. Nevertheless, it seems to be worthwhile to re-address this issue in the future.

2.6. *Functional status of cells and organs*

In forensic pathology main areas of interest are the cause of death and the functional and morphological changes of cells and organs during the processes immediately preceding death rather than etiology and the course of diseases. However, many mechanisms leading to death do not produce morphological changes because they are mainly functional or they do not last long enough to materialize in visible findings. On the other hand, the functional status of a cell is displayed in its mRNA pattern—analyzing this pattern in post-mortem samples therefore could offer insight into the pathological mechanisms leading to death. mRNA up-regulation can occur rapidly within minutes so that even acute events could be monitored.

As pre-requisite for the quantitative use of mRNA analysis on post-mortem tissues it is necessary to establish degradation profiles of selected markers to rule out post-mortem changes of the ratio of marker to control mRNA. Recent studies on erythropoietin, vascular endothelial growth factor and hypoxia-inducible factor 1 alpha mRNA transcripts have demonstrated that these markers which are regulated by oxygen [47] and therefore are possible indicators of hypoxemia, fulfill these requirements and therefore could be used in quantitative assays [48]. Technically microarrays with the potential of analyzing thousands of genes simultaneously offer the best way to assess the mRNA status of a cell or tissue: each cell type possesses its own and unique mRNA pattern dependent on the oxygen and energy supply, transcriptional and translational activity and other cell functions. In theory, a microarray based mRNA scan from autopsy samples could be compared with a database containing known mRNA profiles for various diseases or death mechanisms and could provide a clear-cut diagnosis even without specific morphological findings. Multiplex real time PCR assays lead to the same, although less comprehensive results but require (due to the smaller throughput) a careful selection of markers. The number of published studies dealing with this kind of gene expression profiling in experimental medicine is steadily increasing and it is impossible to give a complete overview at this place. One of the areas with a lot of research activity is brain hypoxia with attempts to identify hypoxia-responsive genes (that is genes suppressed or activated by hypoxia) in order to develop therapeutic strategies to minimize brain damage in ischemic brain disease [49]. It is evident that this might be of great value for forensic pathologists looking for pre-mortem processes leading to death. Other examples involve the co-diagnosis of paraquat-induced lung fibrosis by a spectrum of mRNA markers (mainly cytokines, growth factors and chemokines) as suggested recently [50], the diagnosis of morphine addiction by evaluating the density of μ-opiate receptor mRNA in the brain [51] or the over-expression of heat shock proteins in hypothermia (Madea, personal communication). Molecular changes such as an increase of inducible NO-synthase in certain brain regions also accompany acute alcohol intoxication and might help with diagnosis whenever there is doubt about the interpretation of toxicological results [52]. The molecular changes following mechanical pressure have been already mentioned above. Diagnosis of diffuse axonal injury could be facilitated with molecular markers such as FE65, a beta-amyloid precursor protein-binding protein [53] and diagnosis of traumatic shock with several cytokines and growth factors as markers in lung tissue [54].

Forensic research in this field is at its very beginning with the applicability to human post-mortem tissues still to be shown but thanks to recent studies [48] it is fair to say that this is no longer pure fantasy but beginning to come into the focus of forensic research.

3. Summary

Forensic science encompasses a broad spectrum of different specialties all devoted to the scientifically sound analysis of evidence in criminal cases. Research is an indispensable pre-requisite but heavily depends on the availability and application of up-to-date techniques. A good example is forensic DNA analysis which involved the adaptation of molecular biology methods and knowledge to forensic purposes. Although RNA plays an overwhelming role in modern bio-medical research (and already in diagnostic procedures), it has not gained major attention in the forensic community. This review was aimed to narrow this gap by disclosing technical details and possible forensic applications of RNA analysis in forensic science.

The identification of body fluids by mRNA profiling is an important technique supplementing DNA analysis in cases where the origin and type of evidence is important. A number of markers and assays for all types of body fluids including menstrual blood have been developed and validated and will be soon available for practical use. Due to quantitative analysis methods even negative test results can provide consistent conclusions as long as certain limitations are kept in mind.

Post-mortem RNA degradation is not only time-dependent, but also correlated with other factors such as brain pH, etc. Its use for the determination of the post-mortem interval has been suggested, but certainly requires a broader database covering all possible variations of ambient conditions bodies can be exposed to. One important conclusion from these experiments is that the quantitative composition of mRNA is not likely to change significantly during the first 24–48 h after death which provides

the opportunity to perform quantitative studies on post-mortem material. Determination of the age of wounds and injuries is an application which can be perfectly realized by RNA analysis and which has the potential to become a key method in molecular forensic pathology. Another, more complex prospect is the diagnosis of the cause and mechanism of death and the underlying acute disease by studying pre-mortem changes of gene expression. Using the latest technologies such as real time PCR and microarrays, this could develop into a new era in forensic science and could be relevant for both forensic and clinical medicine.

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