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Original article

A two-step method for the extraction of high-quality RNA from endoscopic biopsies*

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SUMMARY

Background: The usage of molecular techniques such as quantitative RT-PCR depend on the quality of cellular RNA. In particular, RNA extraction from endoscopic biopsies is difficult with respect to yield and, especially, integrity. Therefore, we developed a method that allows extraction of high-quality RNA from these sources.

Methods: Endoscopic biopsies taken from the gastric antrum, corpus and duodenum were subjected to various RNA extraction protocols, and the RNA was used for quantitative RT-PCR.

Results: The subsequent usage of two methods, (i) a phenol/chloroform extraction and (ii) a column-based extraction method resulted in a yield of 4.5µg total RNA/biopsy of reliable quality in 80% of samples. The quantitative RT-PCR analysis revealed that only RNA samples that clearly show both 18S- and 28S-RNA bands in agarose gel electrophoresis were suitable for quantitative RT-PCR. In all these samples, both the corpus-specific pepsinogen C-mRNA and the duodenum-specific mdr-1-mRNA could be consistently detected. In degraded RNA, pepsinogen C, mdr-1 or β-actin mRNAs were still detectable, but the quantitative det4rmination gave inconsistent data.

Conclusions: The two-step method described here proved to be the most reproducible approach to obtaining sufficient amounts of high-quality RNA from endoscopic biopsies as required for quantitative gene expression analyses.

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INTRODUCTION

Gastrointestinal endoscopy is usually completed by taking biopsies from lesions and surrounding tissues. Traditionally, biopsies are used for histological assessment, but molecular analysis of these samples has become a fundamental part of clinical research during recent years.[1] The high sensitivity as well as the high throughput of these methods have offered a comprehensive picture of differential gene expression patterns comparing normal versus pathological situations.[2-4] The usage of high-quality RNA is the most critical factor for quantitative analyses of gene expression patterns in clinical samples which are often 'poor' sources for RNA extraction.[1] In particular, the preparation of RNA from endoscopic biopsies is complicated by the limited amount of available tissue. Therefore, biopsies frequently need to be pooled to increase the yield but this may also level differences in the gene expression. In order to compare the expression levels of different genes by RT-PCR, the expression of 'house-keeping' genes are analyzed and subsequently used to normalize the expression levels among different samples.[5] Since most of these 'standard genes' such as b-actin or GAPDH are present in high copy numbers, they can be detected by RT-PCR even when the RNA is almost completely degraded. In such samples, the expression levels of low- or even medium-abundant transcripts

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might be determined as false negative depending on the extent of degradation. Considering this problem, we developed a methodology allowing for the extraction from endoscopic biopsies of RNA which is suitable for quantitative analyses of the gene expression pattern.

MATERIAL AND METHODS

Sampling and handling of endoscopic biopsies

A total of 25 patients who underwent upper gastrointestinal endoscopy for different indications were included in this study. This study was approved by the local ethics committee. After obtaining informed consent, 2-3 biopsies were taken, pooled into one cryo-tube and snap-frozen in liquid nitrogen. After finishing sample acquisition, each of the 2-3 biopsies were transferred into a 1.5 ml RNAse-free Eppendorf tube and submerged in 0.5 ml of TRIZOL-reagent™ (Life Technologies, USA) and stored at –80˚C until usage.

Extraction of total RNA

A single biopsy in TRIZOL-reagent™ was homogenized using plastic pistils 'DSTROY-S' (BIOzym, Germany) on ice until no particulate material was visible. Samples were subjected to 3-5 freezing (liquid nitrogen) / thawing cycles (ice bath) while treated using the pistil. After complete homogenization, 0.2 ml chloroform was added, the sample was extensively vortexed and centrifuged in a microcentrifuge (14.000 rpm, 4˚C) for 15 min. The supernatant (about 400µl) was transferred to a new tube, 0.4 ml isopropanol was added and the sample was incubated at room temperature for 10 min. After another centrifugation step, the supernatant was completely removed and the precipitated RNA was resolved in 100 µl RNase-free water. Subsequently, the RNA was purified using the RNeasy kit™ (Qiagen, Hilden, Germany) following the protocol described by the manufacturer. Finally, the RNA was eluted in 70 µl RNase-free water, and a 5µl aliquot was used for determination of RNA concentration via UV-spectroscopy and to evaluate RNA integrity by agarose gel electrophoresis.

Reverse Transcription of total RNA

In each case, 500 ng of total RNA was transcribed into cDNA in a 20 µl reaction containing 20 units of AMV reverse transcriptase (Promega, Mannheim, Germany), 0.5 mmol/l dNTP, 10 mmol/l random hexanucleotides (Boehringer, Mannheim, Mannheim, Germany) and 50 units of placenta RNase inhibitor (Ambion, Austin, USA) in the reaction buffer supplied with the enzyme. After an 1h incubation at 42˚C the enzymes were inactivated by a 10 min incubation at 95˚C, and the reaction mixture was kept frozen at -70˚C until enzymatic amplification.

Quantitative RT- PCR

Quantitative RT-PCR was performed in an iCycler (BioRad, Munich, Germany). A typical 30 µl reaction mixture consisted of 15µl HotStarTaqT Master Mix, 1.2 µl of the RT-reaction, 0.3 µl SYBR-Green I (1:10.000) (Molecular Probes, Eugene, USA), and 0.5 µmol/L of the specific primers for b-actin, pepsinogen C or mdr-1. Initial denaturation and activation of Taq-polymerase at 95˚C for 15 min was followed by 40 cycles with denaturation at 94˚C for 30 sec, annealing at 60˚C for 30 sec, and elongation at 72˚C for 30 sec. The fluorescence intensity of the double-strand specific SYBR-Green I, reflecting the amount of actually formed PCR-product, was read real-time at the end of each elongation step. Then specific initial template mRNA amounts were calculated by determining the time point at which the linear increase of sample PCR product started, relative to the corresponding points of a standard curve; these are given as artificial units. b-actin mRNA amounts were used to normalize the cDNA contents of the different samples. Furthermore, an aliquot of the PCR reaction mixture was separated on an 1.8% agarose gel, stained with ethidium bromide and documented.

The following primers were used for the RT-PCR analysis: Pepsinogen C (Genbankδ accession number # J00283), fw: 5'-cca-acc-agg-agt-tcg-gct-tga-g; rev: 5'-gtg-tcc-acg-atg-gcc-tgg-caa-c; Multidrug resistance protein (mdr-1) / P-glycoprotein (PGY-1, accession # M14758.1), primer 1: 5'-agc-aga-gga-tcg-cca-ttg-cac-g, primer 2: 5'-ttg-ggc-ttg-tga-tcc-acg-gac-ac, primer 3: 5'-agg-tga-aga-agggcc-aga-cgc-tg, primer 4: 5'-aca-atg-cag-gtg-cgg-cct-tct-c; b-actin (accession # NM001101) fw: 5'-cat-gcc-atc-ctg-cgt-ctg-gac-c-, rev: 5'-aca-tgg-tgg-tgc-cgc-cag-aca-g. The length of the resulting RT-PCR fragments were 364 bp (pepsinogen C), 428 bp (mdr-1/2), 510 bp (mdr3/4), and 400 bp (b-actin).

RESULTS AND DISCUSSION

Extraction of total RNA from endoscopic biopsies

Analyzing gene expression patterns in endoscopic biopsies requires a methodology that (i) can easily be implemented in the routine endoscopic procedure and (ii) ensures the necessary quality of biological samples. To prevent any mishandling of samples intended for RNA- or protein analysis, all biopsies were equally treated and snap-frozen in liquid nitrogen. The subsequent

resuspension of single biopsies in TRIZOL proved to be favorable. First, this treatment prevented the samples from drying at –80˚C if longer stored, and, second, seemed to have a stabilizing effect on the RNA. As exemplarily shown for six samples (patient 1 and 2 in Fig. 1A), RNA extracted from biopsies stored without TRIZOL for about 6 months was completely degraded, although no major differences in the yield were observed by UV-spectroscopy at 260 nm. Third, the sample was soaked with TRIZOL which effectively destroyed the tissue. Therefore, samples were completely homogenized within 5 min without much effort, whereas samples stored at –80˚C without TRIZOL needed 10 - 15 min of intensive work for complete homogenization. According to our experience, the parallel handling of six samples, that was accomplished within about 2-2.5 h, was efficient regarding the quality and throughput. As shown in the right panel of figure 1A (patient 3 and 4), the majority of RNA samples prepared this way were of good quality as indicated by the presence of 28S- and 18S-rRNA bands. Only the sample extracted from the antrum (A) of patient 4 showed a slight degradation as illustrated by the decreased intensity of the 28S-RNA band.

In general, the two-step protocol described here provided RNA of reliable quality in about 80% of samples. The total yield per biopsy was 4.5 ± 3 μg (1.4 – 12.6 μg, n = 45) which is sufficient for comprehensive gene expression analysis. Although no DNase treatment was performed, RNA samples did not contain significant amounts of contaminating DNA as shown by the usage of intron-spanning primers in RT-PCR analyses (data not shown) or RNA gel electrophoresis. The storage of single biopsies suspended in TRIZOL reagent did not affect the quality of the RNA (up to six months). Initially, we used the TRIZOL-based method and the RNeasy kit (Qiagen) separately, but the use of the kit only resulted in lower yields (usually less than 1 μg). On the other hand, the purity of RNA samples obtained by the TRIZOL method was limited. The combined application of both methods was found to be superior with respect to yield and integrity of the resulting RNA. Unquestionably, this method is more expensive and time-consuming, but these disadvantages are compensated for by the reliability of the method. For other materials that are not limited, easier to handle, or whose sampling can be repeated (e.g. cell lines, surgical material) the RNeasy kit provides similar results at lower costs.

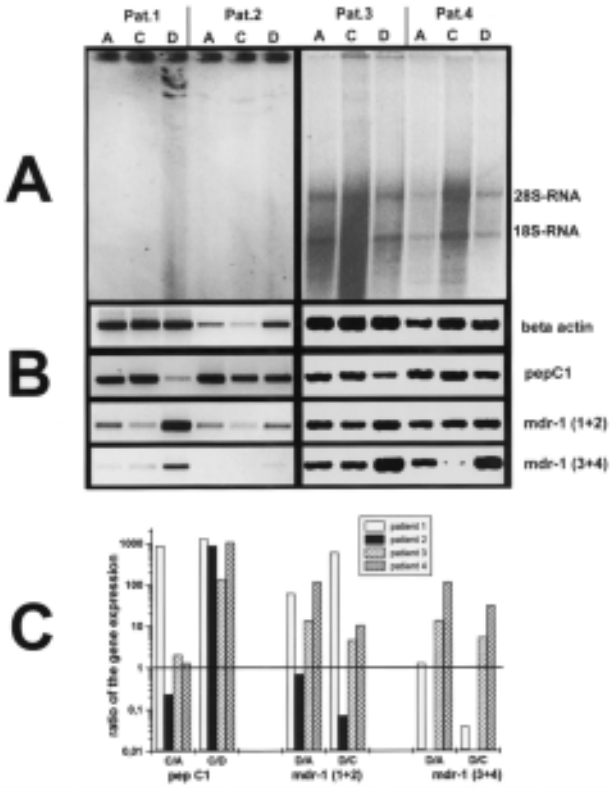

Figure 1. Detection of total RNA isolated from endoscopic biopsies and quantitative RT-PCR analyses of the gene expression of b-actin, pepsinogen C and multi drug resistance protein (mdr-1).

Panel A documents the quality of the RNA extracted from biopsies taken from different regions of the gastrointestinal tract of four patients (antrum: A, corpus: C, duodenum, D). The quality of the six RNA samples (patient 3 and 4) is representative for the extraction method described. Panel B illustrates the PCR products after 40 cycles of amplification. All PCR fragments are of the expected size as calculated from the cDNA sequence (data not shown). Control reactions without template were negative for all analyses (data not shown). Panel C documents the quantitative analysis of the RT-PCR. First, the relative values for either the pepC or mdr-1 transcript were normalized to the corresponding value for b-actin mRNA. Second, using these values the following ratios were calculated and plotted on the Y-axis of the graph: C/A: corpus/antrum, C/D: corpus/duodenum, D/A: duodenum/antrum, D/C: duodenum/corpus. For instance, the normalized pepC-expression rates of patient 1 were 0.59, 521 or 0,4 for the antrum, corpus or duodenum, respectively. Based on these values, the calculated ratios presented in the graph were 880 or 1301 for C/A or C/D, respectively. The horizontal line through the expression ratio of 1 (Y-axes) represents the value of equal expression between the biopsies compared.

The usage of b-actin as internal control and of pepsinogen C and multi drug resistance protein as 'markers' for antrum, corpus and duodenum

In order to determine whether the integrity of the RNA strongly affects the outcome of quantitative gene expression analysis, several intact or degraded RNA samples were used for quantitative RT-PCR. b-actin is part of the cytoskeleton and is ubiquitously expressed in all cells present in the biopsies. Therefore, it was chosen as an internal control, the expression of which was used to normalize expression data determined for other genes. However, it should be noted that there are circumstances where the gene expression of b-actin is subject of regulation itself. Therefore, the b-actin-mRNA level per cell might well differ. Taking into account the current discussion whether 'house-keeping' genes really exist, one should carefully consider different candidate genes (b2-microglobulin, a-tubulin, GAPDH, 28S-rRNA) as internal controls depending on the cellular systems and experimental settings applied.[5-8]

In order to investigate the expression levels of 'marker genes' for the different biopsies [antrum (A), corpus (C) or duodenum (D)], the mRNA amounts of the pepsinogen C gene and the multi drug resistance gene (mdr-1) were analyzed by real-time RT-PCR.

Pepsinogen C (also known as pepsinogen II or progastricsin, E.C. 3.4.23.3) represents the zymogen form of the aspartic protease pepsin C which is synthesized and stored in the chief and mucus neck cells of the gastric mucosa in humans and other mammals.[9] Since these cells are mainly localized in the gastric corpus and, to a smaller extent, in the antrum,[10,11] the transcript level of the pepC gene should be higher in the biopsies taken from the corpus. Furthermore, pepsinogen C-producing cells were identified in the Brunner glands localized in the duodenum, but the overall signal intensity of the immunohistochemical stainings or hybridization signals was much weaker than in the gastric mucosa.[12] Therefore, the amount of the pepC-transcript in duodenal biopsies should be much less than in the samples taken from the gastric antrum or corpus.

The multi drug resistance protein or P-glycoprotein is encoded by the human mdr-1 gene and represents a membrane glycoprotein which is involved in the intestinal drug efflux and mediates resistance to anti-cancer drugs.[13] Localization studies revealed that the mdr-1 protein is widely expressed within the gastrointestinal tract and other tissues showing the highest concentrations in the epithelial cells of the liver and gut.[14-16] Based on this cellular distribution, the levels of mdr-1 transcript determined in the duodenum should considerably exceed the corresponding levels of the gastric antrum or corpus.

Quantitative RT-PCR analysis of pepC and mdr-1 in the gastric corpus, antrum and duodenum

The quantitative RT-PCR analysis presented in Fig. 1B and C revealed some interesting findings. First, regardless of the integrity of the RNA, the b-actin, the pepC, and mdr-1 transcripts were detectable in all samples (Fig. 1B). Second, due to the poor quality of the RNA extracted from patient 1 and 2, the signal intensity of the PCR bands, especially of patient 2, was sharply reduced (Fig. 1B). In particular, this effect was evident for transcripts present at lower levels such as mdr-1 in the gastric corpus or antrum. Third, the usage of a primer set [mdr-1 (3+4)] the binding sites of which were located close to the 3'- end of the mdr-1 transcript barely detected or even failed to detect the corresponding message in the antrum and corpus samples of patient 1 and 2. The fact that this primer set worked well in all samples of patient 3 and 4 strongly suggests that the RNA quality is the critical issue. Fourth, the analysis of PCR products by agarose gel electrophoresis is a simple control for the presence of single PCR bands of the correct size rather than a suitable method allowing for the quantification of differences in the gene expression. Due to the principle of the PCR reaction itself,[17] the quantitative analysis of the amount of PCR products in the stationary phase (end point analyses) will give wrong results in most cases. As an example, the gel electrophoresis revealed only a small difference between the amounts of pepC transcripts detected in the antrum or corpus of patient 1, whereas real-time PCR methodology revealed an 880-fold difference between both samples. The quantitative evaluation was performed for all analyses regardless of the quality of the RNA. For the six RNA samples from patient 3 and 4, the gene expression patterns detected were as expected (Fig. 1C). The biopsies taken from the gastric corpus (C) contained higher pepC transcript levels than that from the antrum (A) and duodenum (D). For the mdr-1 gene, consistent expression ratios were found in all biopsies regardless of the primer set used. Here, the duodenal biopsies exhibited the highest mdr-1 transcript level, followed by the gastric corpus and antrum (Fig. 1C). In contrast, quantitative data obtained for the samples of patients 1 and 2 were not as expected. For instance, patient 1 showed a very high difference (880-fold) in the extent of pepC expression between the gastric corpus and antrum. No duodenal-specific expression of the mdr-1 gene was found using the

distal located primer set (mdr-3+4), whereas the ratios D/A and D/C determined with the primer set 1+2 did only partially correspond to that of patients 3 and 4. The expression ratios determined for samples of patient 2 were strongly obscured, the antrum appeared to contain more pepC transcript than the corpus and the mdr-1 expression in the duodenum was apparently lower than that of the antrum or corpus. Moreover, mdr-1 expression was undetectable using the distal primer set 3+4.

CONCLUSION

Taken together, the data presented clearly show that RNA samples of poor quality are unsuitable for the quantitative analysis of the RT-PCR and can lead to wrong conclusions. Although the specific expression of pepC and mdr-1 was still detectable in the partially degraded RNA samples of patient 1, the unusually high ratio of the pepC expression between the corpus and antrum implied that these values are severely affected by the different extent of RNA degradation in these samples. Considering the enormous experimental work needed for quantitative gene expression studies and the high variability of clinical samples, we strongly recommend an evaluation of RNA-extracting methods with respect to both methodological and sample-related issues. In particular, the RNA integrity of each sample needs to be evaluated by applying agarose-gel electrophoresis. Only samples exhibiting both the 28S- and 18S-rRNA bands should be used for quantitative analysis. The use of samples showing a higher degree of degradation should be limited to qualitative RT-PCR analysis only, or completely omitted. This approach might be helpful to prevent negative surprises after sampling and pitfalls at later stages of analysis. The two-step methodology for the preparation of RNA from endoscopic biopsies described here, has been shown to provide RNA in acceptable yields and of sufficient quality to allow for a comprehensive quantitative expression analysis.

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