Histol Histopathol (2008) 23: 391-395

http://www.hh.um.es

Histology and Histopathology

Cellular and Molecular Biology

Isolation of high quality protein samples from punches of formalin fixed and paraffin embedded tissue blocks

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Summary. In general, it is believed that the extraction of proteins from formalin-fixed paraffin embedded samples is not feasible. However, recently a new technique was developed, presenting the extraction of non-degraded, full length proteins from formalin fixed tissues, usable for western blotting and protein arrays. In the study presented here, we applied this technique to punch biopsies of formalin fixed tissues embedded in paraffin to reduce heterogeneity of the tissue represented in sections, and to ensure analysing mainly defined cellular material. Successful extraction was achieved even from very small samples (0.7 mm^3). Additionally, we were able to detect highly glycosylated proteins and protein modification, such as phosphorylation. Interestingly, with this technique it is feasible to extract high quality proteins from 14 year old samples. In summary, the new technique makes a great pool of material now usable for molecular analysis with high throughput tools.

Key words: Formalin fixed tissues, Protein extraction, Western blotting, Protein modification

Introduction

Fixing tissue with formalin and embedding them in paraffin (FFPE) has been a routine method for decades. By the fixation, proteins are stabilized via cross-linking and cell/tissue structure is mainly preserved. After embedding the samples in paraffin, further processing of the sample to perform histological or immunohistological examination and classification is possible. Multiple reports show that proteins and protein modifications such as phosphorylation are stabilized and can be determined years later by immunohistochemistry (Liotta and Petricoin, 2000; Lim and Elenitoba-Johnson, 2004). However, it was widely expected that these samples were only usable for DNA and RNA extraction but not for proteins. In 1991 Shi et al described for the first time that antigen retrieval by application of heat is feasible (Shi et al., 1991). Recently, an extraction system was developed to extract proteins from formalin fixed paraffin embedded tissues (Ikeda et al., 1998; Chu et al., 2005) and the quality of the samples was evaluated using mass spectrometry (Hood et al., 2005; Shi et al., 2006). First reports on an improved technique, based on the method of Ikeda et al., demonstrated that extraction was effective from tissue sections and that molecules as large as 185 kDa, such as HER2, were detectable in the eluates (Becker et al., 2006).

Extraction of proteins from FFPE tissues has several great advantages compared to kryo-conserved tissues. The most important one is availability, as FFPE is a routine technique all over the world. Laborious infrastructure, such as the need for liquid nitrogen and freezing facilities is not necessary. Additionally, mostly standardized techniques are used for FFPE, helping to ensure high quality of the samples. Also, clinical data are available, e.g. for survival or efficacy of treatment.

Molecular characterization of tumour tissues is greatly needed. Already today there are therapies based on expression changes in tumour tissue. Up to now these target proteins have been quantified by immunohistochemistry (e.g. Her2), which is difficult and rarely standardized. The extraction of proteins would enable us to achieve more accuracy and reproducibility in clinical decisions. With the help of the new protein extraction technique from FFPE samples, additional information can be gained, which is only determinable with great effort by immunohistochemistry. Changes in the molecular size of the protein derived from alternative splicing or mutations can be easily visualized by Western blot, determining changes in the band pattern. In addition to these advantages, several newly developed tools to identify new target molecules, such as protein

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arrays, could be used.

In this study we aimed to reveal whether extraction from punch biopsies is feasible, since extraction from slices has the problem of analysing mixed tissues with parts of stroma and other contaminating cell types, in addition to the presence of cancer cells. As an example, melanoma punch biopsies would result in concentration on tumour tissue with reduced amounts of other cell types. Additionally, we addressed the following points: requirement of cutting the punch into very small pieces; amount of input sample; molecular weight of the proteins and glycosylation; stability of the extracted proteins; age of paraffin blocks and protein phosphorylation.

Material and methods

Extraction of proteins from FFPE tissue

The extractions were performed using the Qproteome FFPE Tissue Kit (Qiagen, Hilden, Germany). The part of tissue that was used for the extraction procedure was crushed with a scalpel and deparaffinized with descending alcohol series in a reaction tube. This was followed by the addition of 100 µl extraction buffer EXB from the Qproteome FFPE Tissue Kit. Samples were incubated for five minutes on ice before incubation of 20 minutes at 100°C and 2 hours at 80°C with gentle agitation. Centrifugation of the sample for 15 minutes at 4°C allowed the transfer of the supernatant into a new collection tube. Competing products which can be used are the Liquid Tissue kit (Expression Pathology).

The following modification to the protocol of the manufacturer was applied: Instead of tissue sections, punches were used in our study. After careful histological evaluation of the samples, the areas representing the required tissue (e.g. tumor) were punched, according to a protocol previously reported for multiple tumor tissue arrays (Wild et al., 2006).

Protein quantification

The protein concentration was determined using the BCA protein assay reagent (Pierce, USA).

SDS Page analysis

Balanced amounts of cell proteins (5 µg) were denatured at 70°C for 10 min after addition of Roti-load-buffer (Roth, Karlsruhe, Germany) and subsequently separated on 10% Page-SDS-gels. Separation was followed by silver staining using the SilverXpress™ Silver Staining kit (Invitrogen), according to the manufacturer's instructions.

Protein analysis in vitro (Western-Blotting)

Balanced amounts of cell proteins (20 µg) were denatured at 70°C for 10min after addition of Roti-load-buffer (Roth, Karlsruhe, Germany) and subsequently separated on 10% Page-SDS-gels (Rothhammer et al., 2007). After transferring the proteins onto PVDF-membranes (BioRad, Richmond, USA), the membranes were blocked in 3% BSA/PBS for 1 hour and incubated with a 1:1000 dilution of primary anti E-cadherin (1:400; Takara), anti MIA2 (1:300; (Hellerbrand et al., 2005)), anti-ERK1/2, anti P-ERK1/2 (1:1000; Cell Signaling), or ß-actin (1:5000, Sigma) overnight at 4°C. Anti-rabbit or mouse-AP (1:2000 or 1:4000; Sigma) were used as secondary antibodies. Staining was performed using NBT/BCIP solution (Zytomed, Heidelberg, Germany). All experiments were repeated at least three times with similar results.

Statistical analysis

Results are expressed as mean ± SD (range) or percent. Comparison between groups was made using the Student's unpaired t-test. A p value <0.05 was considered statistically significant. All calculations were performed by using the Graph Pad Prism software (Graph Pad software, San Diego, USA).

Results

Protein extraction was performed from tissue samples of normal liver and malignant melanomas. In contrast to the established protocol, in which proteins were extracted from tissue sections, punches were used, as later defined tumour areas are aimed to be analysed. We started using a punch with diameter 1mm, using punches of approximately 4.5 mm length (=3.5 mm^3). This equals the suggested amount of material. Here, formalin fixed liver samples were used which were freshly prepared to rule out the effects of modifications by fixation and age of sample in this first analysis. Using paraffin punches (Fig. 1A) it was shown that crushing the material was essential for effective isolation. This was estimated measuring the amount of isolated proteins (Fig. 1B) and the quality of the samples in SDS-page followed by silver staining (Fig. 1C). Here, the maximal protein amount was isolated using extensive crushing of the sample material. In addition, the quality of the extracted material was optimal, as determined by defined bands in the SDS-page gel. In general, extraction of tissue was very efficient. Using one punch 1.5-2.23 µg/µl protein were isolated. To verify reproducibility, three punches out of one liver sample were analysed in parallel. The range of extracted protein was 1.43 to 1.76 µg/µl, revealing high reproducibility.

As laser microdissection could be potentially used to isolate proteins of defined tissue areas, we tried to minimize the amount of sample needed. We reduced the size of the punch from the liver sample using 1/4 (0.25) of the punch as the smallest (approx. 0.7 mm^3). All assays were repeated three times. Even with the smallest

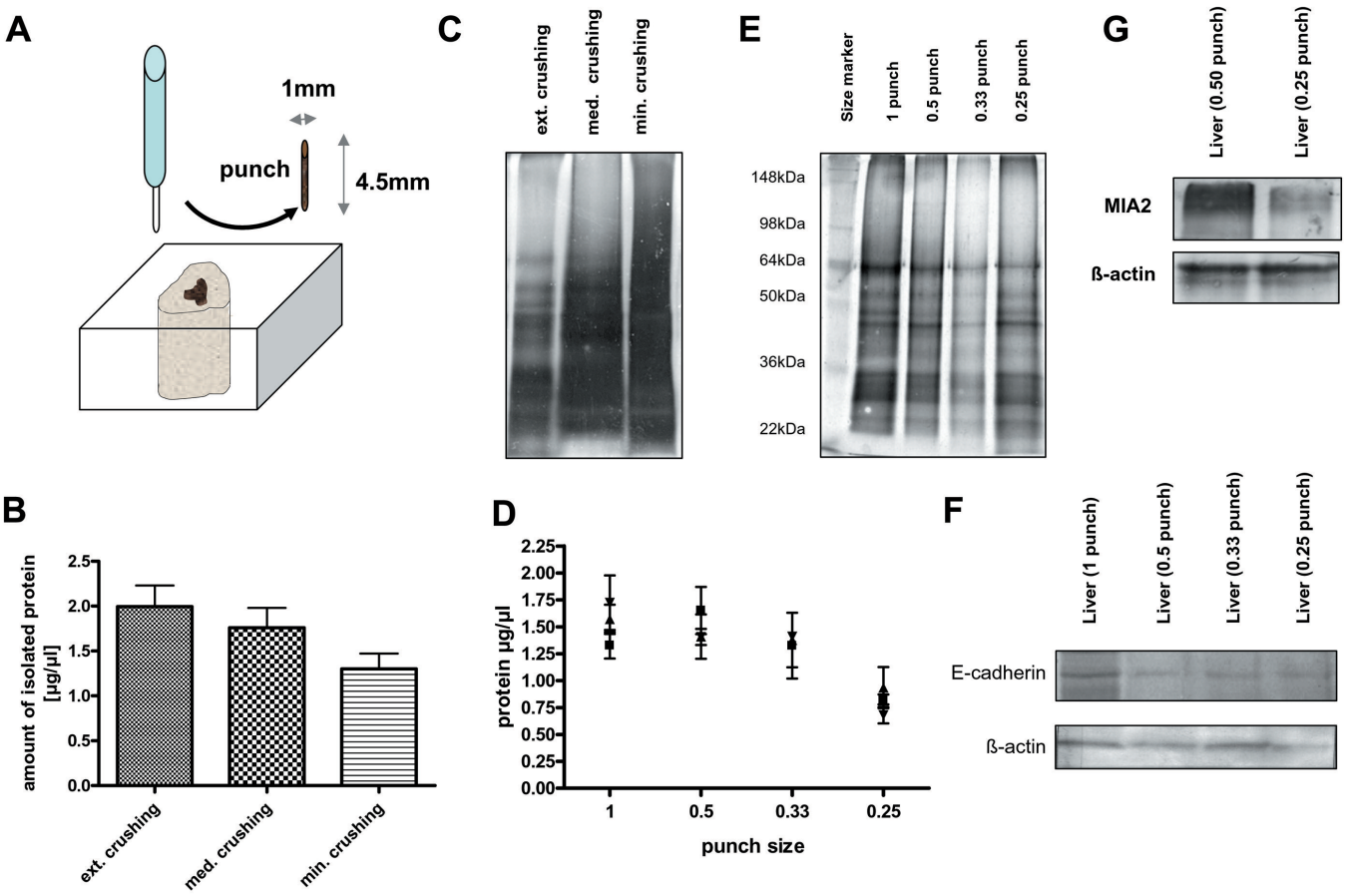

Fig. 1. Amount of protein extracted from punches. Punches of the same size from three different liver samples were used to extract protein. The crushing of the sample prior to extraction was varied from extensive (ext.), medium (med.) to minimal (min.). The amount of protein after extraction was measured by BCA assay **(B)**. The samples were additionally analysed by SDS-Page. In **C** one representative example is shown. Punches of 4 different sizes from three different liver samples were used to extract protein. The amount of protein after extraction was measured by BCA assay **(D)**. A linear correlation was found between sample size and amount of protein obtained, focussing on sample size 0.5 to 0.25 (r^2=0.820). The samples were additionally analysed by SDS-Page. In **E** one representative example is shown confirming the quality of the extracted proteins. Different punch sizes of tissue samples of formalin fixed paraffin embedded normal liver were analysed. Balanced amounts of extracted protein were subjected to SDS-Page followed by western blotting. Membranes were stained with anti-E-cadherin and anti-MIA2 antibody, respectively **(F, G)**.

amount of sample, sufficient amount of protein was obtained (Fig. 1D). It seems that using one complete punch the capacity of isolation was saturated. A clear linear correlation (r^2=0.820) between punch size and amount of extracted protein was observed using 0.5 to 0.25 punches. Analysing the extracted proteins on SDS-page followed by silver staining revealed the high quality of the extracted proteins in all samples (Fig. 1E). Looking at the images we got the impression that isolation of larger proteins (>70kDa) could be problematic.

To analyse the extraction of large proteins from paraffin, either the liver-specific molecule MIA2 (glycosylated MIA2: >85kDa) or E-cadherin (110kDa) was chosen. E-cadherin was detectable in liver samples using 1 to 0.33 punch but only weakly in the smallest amount of sample material (0.25 punch) (Fig. 1F).

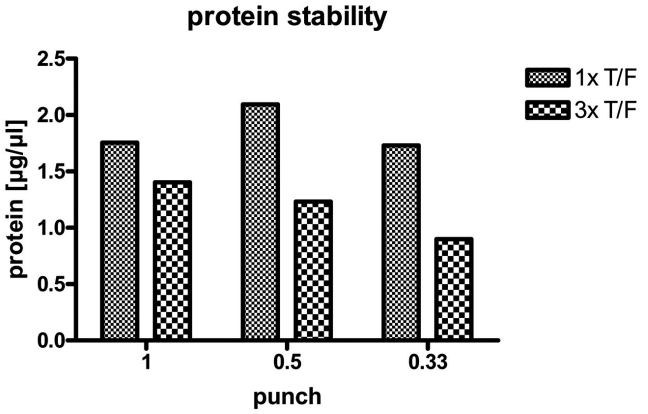

Fig. 2. Stability of isolated proteins. The extracted protein samples were subjected to repeated freezing and thawing cycles (T/F) followed by determination of the protein amount via BCA assay.

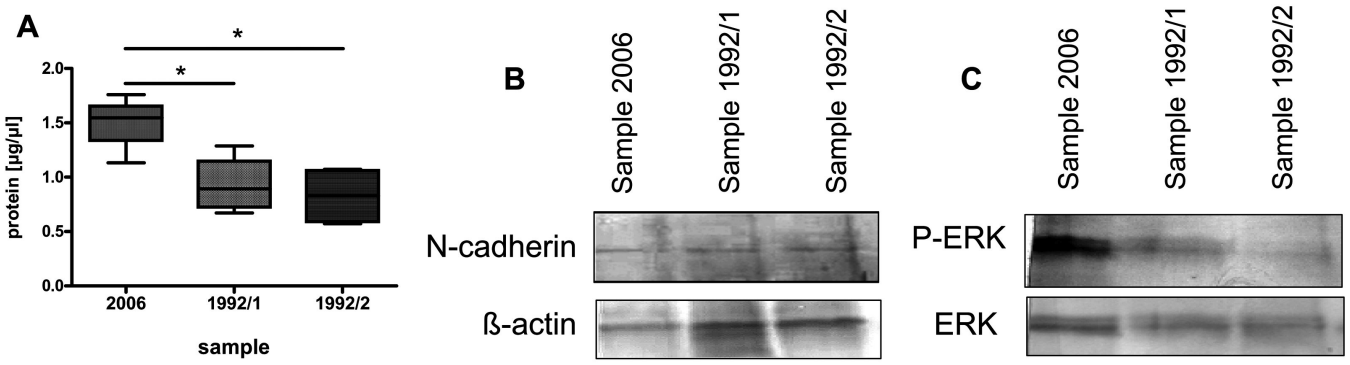

Fig. 3. Impact of sample age. Proteins were isolated from two independent 14 year old samples (sample 1992/1 and 1992/2) and compared to extractions from a sample only recently generated (sample 2006). Each extraction was repeated four times. **A.** The amount of extracted protein was measured using the BCA assay. A significant reduction in the amount of extractable protein was observed (*: $p<0.05$). Western blotting was performed for N-cadherin and ß-actin **(B)**, or phosphorylation of ERK and ERK **(C)** were stained.

Looking at MIA2 in the smallest sample size, protein was detectable (Fig. 1G), although also reduced compared to ß-actin. These assays revealed that isolation of large proteins also with strong modifications, like the heavy glycosylated molecule MIA2, is feasible. However, a certain amount of sample material is necessary.

We also analysed the stability of the protein in the extraction solution after 1 or 3 freezing/thawing (T/F) cycles. A strong reduction of total protein was determined after the cycles (Fig. 2). In laboratory use, therefore, aliquots should be made immediately after the extraction.

It is extremely important that this new method for protein extraction is not only feasible for recent samples but also for older material in the archives. We therefore used FFPE melanoma tissues which were 14 years old to define the potential of the new method. Proteins were isolated from two independent 14 year old samples (sample 1992/1 and 1992/2) of malignant melanoma and compared to extractions from a sample only recently generated (sample 2006). Each extraction was repeated four times. A significant reduction in the amount of extractable protein was observed (Fig. 3A). Analysing the quality of the preparation revealed that N-cadherin and phosphorylation of ERK was detectable in all samples (Fig. 3B,C). This finding shows that stability of proteins is ensured by the fixation and even modifications are kept.

Discussion

In this study we revealed that using punch biopsies in contrast to sections of the FFPE material is feasible for the extraction. A little more background staining is seen in the Western blots compared to the protein analysis from e.g. frozen tissue sections. We found that thorough homogenisation of the material is necessary to obtain a high quality preparation of the proteins. The amount of material can be strongly reduced without lowering the quality. However, isolation of larger proteins seems to be not as effective as that of proteins smaller than 70kDa, and these can be disproportionately reduced or lost using very small sample sizes. Possibly, the volume of the protein extraction buffer needs to be adjusted; i.e. less than 100 µl extraction buffer may be used for protein extraction from small samples.

Even older samples can be used, showing a reduction in the amount of extracted protein but no changes like degradation products. Additionally, modifications of the proteins are conserved. However, as it is difficult to determine the stability of post-translational modification, we suggest comparing only samples of similar storage time.

After extraction it is absolutely necessary to confirm the quantity and quality of the isolate by SDS-page followed by silver staining. Additionally, before analysing these samples, the specific antibodies which are going to be used have first to be tested on these samples. This is to ensure that the antigenic sites are not modified or destroyed by the fixation or protein isolation. Furthermore, successful isolation of the protein to be analysed has to be determined previous to larger studies, especially if it is a large molecule or heavily modified.

In summary, we were able to show that this method is suitable for extracting proteins from formalin fixed paraffin embedded tissues with high quality, and conservation of posttranslational modifications. As formalin fixed paraffin embedded tissues are broadly available, this new method can mean progress in determining and analysing molecules in physiological and pathological situations.

Acknowledgements. We are indebted to Susanne Wallner for excellent technical assistance. This study was supported in part by the German Research Society (DFG) to KFB and to AKB .

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Accepted September 26, 2007