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MET, HGF, EGFR, and PXN gene copy number in lung cancer using DNA extracts from FFPE archival samples, and prognostic significance

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Abstract

Gene copy number analysis for some of the important molecules in lung tumorigenesis such as MET, hepatocyte growth factor (HGF, ligand for MET), epidermal growth factor receptor (EGFR) and paxillin (PXN) is likely to determine both the type of treatment and prognosis. Formalin-fixed paraffin-embedded (FFPE) archival tumor tissue samples are an excellent source for determining key molecular changes in the OncoGenome, however existing extraction procedures yield relatively poor quality genomic DNA fragments. Although FISH is the method of choice for determining amplification of a gene, a more rapid qPCR technique to determine gene copy number can be used when reasonably good quality genomic DNA is available. We report here a relatively rapid method based on microwave/chelex-100 treatment that gives rise to genomic DNA fragments ranging from 1 to 12 Kb and beyond, thereby attesting to its superior quality. Genomic PCR for β -globin gene gave reliable and reproducible results. The number of steps for extracting the DNA was kept to a minimum, and instead of precipitating the DNA, we preserved the genomic DNA extracts so as to prevent a loss in DNA yield. We found the extracts to be stable and amenable to qPCR and mutational analysis. Using lung adenocarcinoma FFPE samples and cell lines derived from lung adenocarcinomas, we demonstrated that the gene copy number for MET in lung adenocarcinoma tissue samples was preferentially increased over EGFR, HGF and PXN and that it positively correlated with better prognosis. In contrast, the genomic DNA extracted from twenty five NSCLC cell lines gave relatively higher gene copy number for all the four genes evaluated. Our results indicate that the microwave/chelex-100 based method yields good quality genomic DNA extracts that can be used for complex DNA analysis such as determination of gene copy number. In addition, our data demonstrated that the adenocarcinoma cell lines potentially evolved under ex vivo conditions, and therefore in genetic studies it is imperative to use primary tumors for generalized conclusions about lung tumors.

Keywords

Genomic DNA; FFPE; MET; EGFR; Paxillin; Gene copy number

Introduction

During the year 2008, there were about 215,000 new patients diagnosed with lung cancer, more than 80% of whom will die of their disease. Despite tremendous advances in our understanding of cancer biology and developments in cancer therapeutics, the prognosis for lung cancers

remains extremely poor [1]. One solution lies in understanding the lung cancer genome and its variations in their entirety, based not only on the histologic type, but also the developmental stages of lung cancers. One of the best resources to study the lung cancer OncoGenome is the formalin-fixed paraffin-embedded (FFPE) archival samples. FFPE tumor tissues serve as indispensable tools for determining proteinaceous antigens and therefore serve the cause of research, diagnosis and teaching.

Embedding FFPE tumor tissues facilitates thin sectioning for antigenic analysis. The current commonly used immune-histochemical procedures owe their existence to the earlier discovery of boiling the FFPE samples to eliminate paraffin from the sections [2–3]. Several variations to the above procedure have been introduced that involve xylene extraction at relatively high temperatures (55°C) for deparaffinization of the samples followed by several alcohol washes that abolish any traces of xylene [4–5]. A variation to the above procedure is the use of microwave energy for the purpose of deparaffinization (6–7)

Several methods exist to isolate genomic DNA from FFPE samples. However, due to the miniscule sample size, formalin treatment, and variations in pH, the DNA yields are not only extremely low, but the quality of the DNA is very poor. The isolated genomic DNA fragments are usually less than 500 bp and therefore are very likely to add to significant errors in DNA analysis [8]. These are the major hurdles that limit the use of archival tumor FFPE samples for genomic DNA analysis. We report here an alternate minimal procedure that eliminates several steps and combines previously reported microwave treatment for deparaffinization and chelex-100 treatment for the elimination of multi-valent metal ions that invariably accelerate DNA degradation and inhibit amplification of DNA by PCR [9–11]. In addition, we circumvent the genomic DNA precipitation step that invariably results in a significant loss in the yield and preserve the processed sample as an aqueous extract for future use.

We compare the widely used xylene extraction procedure with our modified microwave/chelex-100 method and demonstrate that both are equivalent; our method has the added advantage of eliminating the use of xylene and DNA precipitation steps. The method is not only rapid and less expensive; it also yields genomic DNA fragments whose molecular weight far exceeds 500 bp. In addition, the DNA is amenable to mutational analysis, and qPCR and gene copy number assessment. We also report here, using genomic qPCR, the gene copy numbers for some of the important molecules in lung adenocarcinoma such as the receptor tyrosine kinases MET and EGFR, the ligand HGF for MET, and the intracellular signaling molecule PXN using formalin-fixed tissues and cell lines. We demonstrate a difference in gene copy numbers comparing formalin fixed tissues to cell lines, suggesting that cell lines potentially evolve under ex vivo conditions. Finally, we provide unambiguous evidence that demonstrates a preferential increase in the gene copy number of MET compared to EGFR or PXN in lung adenocarcinomas, and show that the MET gene copy number can be used as a prognostic marker for NSCLC.

Materials and Methods

Fresh frozen lung tumor tissues, FFPE lung tumor blocks and lung cancer cell lines-Sample preparation

The archival FFPE lung adenocarcinoma samples were obtained as per approved IRB protocol, and the lung adenocarcinoma cell lines were from ATCC. The microtome blade, tweezers and other equipment that came in contact with the FFPE were all sterilized. For each new FFPE block the equipment was cleaned between the samples and the blade was changed. The cutting surfaces of the microtome were covered with sterile plastic film on which the sections were collected. Gloves were changed for every new block of FFPE.

Aqueous extracts of DNA from fresh frozen human lung adenocarcinoma tissues and cell lines

Fresh frozen tissue samples (50–100mg) were ground in liquid nitrogen using sterilized mortar and pestle and the genomic DNA was extracted using 'Easy DNA' kit from Invitrogen (Carlsbad, CA) as per manufacturer's protocol. Lung cancer cell lines were cultured and 10×10^6 cells were lysed using the above kit and the genomic DNA was prepared.

Aqueous extracts of DNA from FFPE sections using xylene

The FFPE tumor sections (3–4, each 10 μ m thick) were extracted with xylene (1.0 mL) and incubated at 60° C for 20 min with shaking. The samples were centrifuged, the xylene was removed, and the step repeated. Traces of xylene were removed by successively washing with 100%, 95%, and 70% ethanol. The samples were then dried at 37°C for 15min and resuspended in digestion buffer [50mM Tris pH 8.5, 1mM EDTA, 1% Tween-20] and deproteinized with proteinase K (500kg/ml) for 48h at 55°C They were then boiled at 95°C for 10 min and stored at –70°C for future use.

Aqueous extracts of DNA from FFPE sections using microwave and chelex-100

A similar number of FFPE sections in parallel were suspended in digestion buffer and subjected to microwave irradiation for six cycles, each 15sec at high power They were then centrifuged [12,000 $\times$ g, 10min] and placed on ice. The solid wax ring was removed using a sterile toothpick. The samples were then deproteinized with proteinase K (500 μ g/ml) for 48h at 55°C. CHELEX-100 was added (5% slurry, final) to remove divalent cations in the sample extracts that are known to interfere with PCR procedures and accelerate DNA degradation [9–11]. The proteinase K was inactivated by incubating the samples at 95°C for 10 min. They were then centrifuged and extracts preserved at 70°C till further use.

β -globin PCR

We used the forward (ATGGTGCACCTGACTCCTGAGG) and the reverse (GCCATCACTAAAGGCACCGACC) primers to determine the integrity of the β -globin gene in DNA extracts prepared by the above two methods. The DNA denaturation was carried out at 95°C at 5min followed by 35 PCR cycles [95°C, 45sec; 55°C, 45sec; 72°C, 1min] that ended with a final extension at 72°C for 10min.

Gene copy number using QPCR

The forward and reverse primers used for MET, EGFR, HGF and PXN genes are given in Table1 and the procedure used has been previously described [10].

Statistical techniques

Gene copy numbers were compared using ANOVA and unpaired t-tests incorporating a correction factor for multiple comparisons. The frequency with which increased gene expression (>4 copies) occurred was compared using chi square analysis. Kaplan-Meier survival was calculated comparing patients with and without gene expression exceeding 4 copies for each gene.

Results

Genomic DNA extraction from fresh lung tumor tissues and cell lines using a commercial kit

We initially used the commercially available Easy DNA kit to isolate genomic DNA from seven lung cancer cell lines (Fig. 1A, lanes 1–7) and four fresh frozen lung tumor samples (Fig. 1B, lanes 8–11). The isolated genomic DNA was analyzed by agarose gel electrophoresis to

determine the size of the DNA fragment and the results are shown in Fig. 1. The DNA spreads for cell lines and fresh tumor samples were comparable. Most of the genomic DNA migrated as a high molecular weight band (>12 Kb) as expected since the samples were fresh, and the starting amounts of tissue samples and the number of cells from the seven lung cancer cell lines were optimum. In order to further assess the quality of the extracted DNA, we also carried out genomic PCR to amplify a fragment specific for the β -globin gene. As shown in Fig. 1C, we amplified a single 355 bp DNA fragment from all the eleven samples that represented the β -globin gene. Although the above method was successful in isolating genomic DNA of relatively high quality, it failed to yield a reliable quality and quantity of genomic DNA from the available limited quantities of lung tumor FFPE sections (data not shown). In addition, we needed a procedure that was capable of efficiently stripping the paraffin from the archival FFPE lung adenocarcinoma tumor sections. We therefore compared a previously reported xylene based method and our own procedure that is based on the combined use of microwaves to liquefy paraffin and chelex-100, a chelator of heavy metals that invariably contaminates the isolated genomic DNA and interferes with subsequent DNA manipulations such as PCR.

Comparison of xylene and microwave/chelex-100 based genomic DNA extraction procedures from FFPE lung tumor sections

The genomic DNA extracts obtained from three FFPE lung adenocarcinoma samples were analyzed by agarose gel electrophoresis to assess the size of DNA fragments and the results are presented in Fig. 2. Both the xylene and microwave/chelex-100 based methods yielded similarly long genomic DNA fragments that extended from 1 Kb to 12 Kb and beyond (Fig. 2A, compare lanes 1, 2, 3 respectively with lanes 4, 5, 6). The quality of the extracted genomic DNA was superior to what has been reported in the literature using FFPE samples. In addition, using the above genomic DNA samples, we successfully amplified the 355 bp fragment of β -globin gene (Fig. 2B), further attesting to the superior quality of extracted genomic DNA. Despite the fact both methods yielded similar results, in order to avoid the exposure to and constant handling of toxic organic solvent such as xylene, we chose to use the more environmentally friendly microwave/chelex-100 method for subsequent analysis of lung adenocarcinoma cell lines and adenocarcinoma FFPE archival samples.

Gene copy number of MET, HGF, EGFR and PXN in lung NSCLC using microware/chelex-100 method

In order to further evaluate the quality of genomic DNA extract, we processed several archival lung adenocarcinoma FFPE samples using the microwave/chelex-100 method and determined the gene copy number of select genes that are known to play a significant role in lung tumorigenesis using qPCR [12]. The individual data points obtained for MET, EGFR, HGF, and PXN gene copy number for the lung adenocarcinoma FFPE and cell lines are shown in Figures 3A and B, respectively, and the data are summarized in Table 2. We observed a significantly greater increase in the gene copy number of MET compared to HGF, EGFR and PXN. For FFPE tissue, the mean gene copy number was different among the four genes (ANOVA; $p < 0.001$); the MET mean copy number was greater than the mean copy number for EGFR, HGF, and PXN by unpaired t-test ($p < 0.01$ for each; Table 2). There were no differences among the other genes in mean copy numbers. For cell lines, the mean gene copy number was different among the four genes (ANOVA; $p = 0.005$); however, there were no significant differences among the genes in mean copy numbers. For FFPE tissue, the frequency of copy numbers exceeding 4 was greater for MET than for the other genes ($p < 0.001$). For cell lines the frequency of copy numbers exceeding 4 was greater for EGFR than for PXN ($p = 0.03$) and for HGF ($P = 0.012$). Comparing FFPE samples and cell lines, the frequency of increased gene numbers for MET was lower in the cell lines ($p = 0.007$) whereas the frequency was higher for EGFR in the cell lines ($p < 0.001$; Table 2). Long-term survival was improved for patients with

MET overexpression compared to those without (Fig. 4); there were no survival differences among patients with and without overexpression for the other 3 genes.

Discussion

One of our long term goal is to document molecular changes in the lung OncoGenome that significantly contributes to the tumor development and metastasis. A rich source for OncoGenome analysis is the archival lung tumor FFPE sample library that we have initiated and added to over the years. The commercially available protocols and kits for processing and obtaining the DNA required for analysis require large sample sizes. In order to facilitate speedy processing and handling of the relatively small sample size from FFPE sections we developed a modified method for genomic DNA that combines the use of microwave and chelex-100 based methodologies. In order to avoid loss of DNA and decrease the amount of time required for processing each sample, we completely avoided DNA precipitation and washing steps. Here we showed that the xylene based method as well as our modified procedure that combined microwave and chelex-100 steps yielded DNA fragments that were greater than 1 kb. The range of DNA fragments obtained for freshly grown NSCLC cell line samples and that of archival lung adenocarcinoma FFPE sections were comparable for both methods. The amounts of genomic DNA fragment specific for β -globin gene amplified by procedures were also comparable.

The most commonly used procedure to isolate genomic DNA from archival FFPE samples is based on hot xylene extraction for the removal of paraffin, followed by phenol/chloroform extraction for deproteinization [8]. A recently published procedure has replaced xylene with microwave irradiation [6–7]. In the xylene as well as the microwave irradiation methods used here, instead of phenol/chloroform extraction, we used exhaustive digestion with proteinase K for deproteinization. In order to improve genomic DNA yield, we completely eliminated DNA precipitation and washing procedures and also avoided adding carrier agents such as glycogen. Since the DNA extracts and preparations are usually contaminated with heavy metals that are known to dampen and hinder PCR, we modified the microwave method by including chelex-100 in our procedure [11]). Although the commercial procedure in this study yielded relatively high molecular weight genomic DNA fragments from fresh tissue (Fig. 1), we could not adopt this method to FFPE samples mainly due to limited sample availability. In addition, we obtained very similar results using both xylene and microwave/chelex-100 method, however we chose the latter method due its ease and complete avoidance of toxic organic solvents. Since precipitation and washing steps resulted in significant loss in DNA yield (data not shown) and since the next step involved PCR, we extracted the genomic DNA into TE buffer. We also compared chloroform/phenol extraction with proteinase K digestion. The former step however resulted in significant loss in the DNA yields and therefore we chose to adhere to proteinase K digestion (data not shown). The microwave/chelex-100 method we describe here is less labor intense and is relatively rapid, if we were to exclude the 48 h of idle incubation time used up for proteinase K digestion. In comparison to xylene extraction method, our modified microwave irradiation procedure has minimum number of steps and therefore less prone to errors and more likely to give higher yields (data not shown).

Pre-fixation factors such as tissue type and amount, and the kind of fixative used are known to contribute to variations in the quality and yield of genomic DNA. We however used similar amounts of FFPE lung adenocarcinoma cells thereby eliminating the above variables. The variations reportedly due to different storage times of the extracted FFPE samples however were not apparent in the three adenocarcinoma samples we compared (Fig. 2). The fact that we obtained similar DNA spreads on agarose gel and comparable amplified amounts of β -globin gene specific fragment attest to the quality of DNA extracts.

The genomic DNA isolated from fresh NSCLC cells or tumor biopsies yielded more uniform high molecular weight genomic DNA fragments. This clearly suggested that the formaldehyde fixation procedure used to preserve tumor samples is the underlying factor for the observed lower molecular weight DNA fragments (compare Fig. 2 to Fig. 1). However, when compared to previously published procedures that reported average genomic DNA fragment size ≤ 500 bp (), both the xylene and the microwave/chelex-100 methods we adapted here yielded fragments that were clearly ≥ 1.0 kb. The superior quality of the extracted DNA is due to minimum number of steps in our procedure and the replacement of chloroform/phenol extraction by proteinase K digestion. Similar amounts of the amplified β -globin gene product from every sample tested demonstrated that the quality of genomic DNA extracts prepared using the microwave/chelex-100 method were comparable to those obtained using the xylene based method (Fig. 2). We therefore used the microwave/chelex-100 procedure to prepare genomic DNA extracts from FFPE NSCLC sections that were then used to determine gene copy number of some of the key genes known to play a significant role in lung tumorigenesis.

We and others have shown that receptor tyrosine kinases such as EGFR and MET significantly contribute to the growth and metastasis of variety of cancers including lung cancer [13–14]. We have previously reported that MET is overexpressed in both NSCLC and SCLC and that the expressed form is constitutively active [15–16]. In addition, MET gene is known to be frequently mutated in lung cancers in the extracellular ligand-binding SEMA domain and the regulatory juxtamembrane domain; and both types of mutations appear to confer gain-of-function [16]. In contrast, the gain-of-function mutations in EGFR were restricted to the kinase domain. Gefitinib and erlotinib, EGFR kinase inhibitors, are being currently used with relatively high success on targeted lung cancer patients. The SU11274 and PHA665752 compounds that specifically inhibit MET kinase are in clinical trials [17–18]. Surprisingly, lung tumors of East Asian but not Caucasian origin appear to over express EGFR and undergo frequent mutations [19–20]. In addition it has been demonstrated that induction of resistance to gefitinib in a lung cancer cell line resulted in the focal amplification of MET thereby suggesting that MET is likely to contribute to the resistance seen in cancer patients treated with gefitinib [21–22]. We also showed that the intracellular signaling adapter molecule paxillin is overexpressed in NSCLC and frequently mutated; and is a significant contributor to lung tumorigenesis [11].

Since increased gene copy number could be the underlying mechanism resulting in the overexpression of the above proteins in lung cancer, using qPCR, we determined the gene copy number of four genes: MET, HGF, EGFR and PXN. The present study demonstrated a significant increase in the gene copy number of MET over the other three genes in archival lung adenocarcinoma samples. In contrast, the lung adenocarcinoma cell lines revealed a preferential increase in the gene copy number of EGFR over other genes. PXN and HGF gene copy numbers were relatively low compared to MET and EGFR in both lung tumor samples and cell lines.

The preferential increase in the MET gene copy number stands in contrast to a previously reported study on EGFR [23]. Bunn and coworkers recently estimated that 32% of the NSCLC tumors had significantly higher gene copy number for EGFR [23]. Their study was based on FISH analysis whereas we used qPCR. In future, we plan to compare both the methods to further validate the procedure used here. One striking result we obtained was that the gene copy number for MET was significantly elevated in lung adenocarcinomas and the EGFR copy number was preferentially increased in cell lines. The fact that PXN and HGF gene copy number were close to normal (2n) in NSCLC adenocarcinoma samples suggested that our method indeed is reliable. The increased gene copy number and expression of EGFR frequently seen in lung tumors has been correlated to better prognosis [24], whereas in this study we clearly demonstrated the elevated MET gene copy number associated with NSCLC was in

retrospect analysis positively correlated better prognosis. In conclusion, we propose the use of microwave/chelex-100 method for genomic DNA extraction from archival FFPE tissue samples. The extracted DNA can be preserved as such and is amenable to complex genome analysis such as determination of gene copy number. We also demonstrate here striking differences between lung adenocarcinoma tumor tissues and cell lines in terms of gene copy numbers of both MET and EGFR. Taken together, our results question the validity of gene copy number analysis in cell lines suggesting that ex vivo culture conditions can result in the acquisition of genomic alterations that may not reflect the in vivo situation and therefore warranting a more careful selection of model cell lines.

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Fig. 1. PCR based amplification of β -globin gene segment extracted from lung cancer cell lines and fresh tumor tissues using Easy DNA kit

A. Agarose gel electrophoresis of genomic DNA extract from various lung cancer cells and **B.** fresh frozen tumor biopsy samples. **C.** β -Globin PCR from above extracts (lanes 1–7 represent lung cancer cell line samples; and lanes 8–11, fresh frozen lung tumor tissues).

Fig. 2. Xylene and modified microwave methods of genomic DNA extraction

A. Comparison of genomic DNA spread from three FFPE lung tumor samples by the two methods using agarose gel electrophoresis. **B.** β -Globin PCR of the above two sets of samples.

Fig. 3. Gene copy number of MET, HGF, PXN, and EGFR in NSCLC

The gene copy number of MET and its ligand HGF, EGFR, and the adapter paxillin (PXN) were determined by QPCR. The gene copy number for the above genes for each sample is shown for NSCLC archival tissues (A) and cell lines (B).

Fig. 4. Relationship between MET gene copy number and long term survival of NSCLC patients
Kaplan-Meier survival curves for patients with >4 MET gene copy numbers (median 39 months) and those with <4 MET gene copy numbers (median 16 months; $p=0.06$; hazard ratio 0.32; 95% confidence interval 0.02 to 1.09).

Table 1

Primers used in qPCR

MET	Fwd: 5-GCT GGT GGT CCT ACC ATA CAT G-3
MET	Rev: 5-CTG GCT TAC AGC TAG TTT GCCA-3
Line-1	Fwd: 5-AAA GCC GCT CAA CTA CAT GG-3
Line-1	Rev: 5-TGC TTT GAA TGC GTC CCA GAG-3
PXN	Fwd: 5-TCT TCG AGC GGG ATG GAC-3
PXN	Rev: 5-GCC GGA TGA GGA ACT CAC AT-3
EGFR	Fwd: 5-GAA TTG GGA TGA AGA GCT TC-3
EGFR	Rev: 5-GAC ATG CTG CGG GTG TTT TC-3
HGF	Fwd 5-CTT AAA GCC TTG CCA ACA GC-3
HGF	Rev: 5-GCC TAG CCA TGC TCT TTC-3

Table 2**Copy number of MET, Paxillin, EGFR and HGF in lung adenocarcinoma tissues and cell lines**

The values represent mean gene copy number $\pm$ SEM, with number of samples in parenthesis. The percentages indicate the frequency of occurrence of gene copy number ≥ 4 .

GENE	Lung AD (FFPE)	Lung AD (Cell Lines)	P value
MET >4 copies	5 $\pm$ 1.5 (19) 84%	5 $\pm$ 5.1 (25) 40%	0.87 0.007
PXN >4 copies	3 $\pm$ 1.0 (19) 11%	4 $\pm$ 2.0 (25) 28%	0.11 0.16
EGFR >4 copies	2 $\pm$ 1.1 (19) 5%	10 $\pm$ 12.4 (25) 72%	0.007 <0.001
HGF >4 copies	2 $\pm$ 1.2 (19) 11%	4 $\pm$ 1.7 (25) 32%	0.011 0.09