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view

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KRAS Mutations in Historical Tumour Specimens of the Viennese Museum of Pathological Anatomy

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Sir:

The Viennese museum of pathological anatomy displays worldwide one of the biggest collection of specimens. It comprises more than 50 000 exhibits including about 27000 formalin fixed samples of a variety of diseased organs. This collection comprises primarily extraordinary and unique objects, which were used mainly for presentation and exhibitions. While palaeopathologic studies on ancient material are common, only few studies dealt with historical specimens from the 19th century ¹⁻³. Although ancient DNA samples of tumours have been studied ⁴, historical tumour exhibits, to the best of our knowledge, have not been investigated yet. We thus focused on tumours of the 19th and early 20th century in order to look for relevant mutations that developed under different environmental conditions.

Thirty-one tumour specimens were selected ranging in age from 79 to 194 years. Five slices (5 µm) of paraffin embedded tissue were treated with ethanol and incubated overnight to digest proteins. Finally, DNA was precipitated, washed, dissolved, and stored at -20°C until PCR analysis. As photometric analysis was not feasible due to the small yield of isolated gDNA, a PCR method has been developed with the aim to document the length of residual gDNA present in the specimen. As approach, a multiplex PCR assay based on the β-actin gene was developed focusing on four different fragment lengths (120 bp, 175 bp, 365 bp, and 593 bp). To generate the desired fragments, four forward primers and three reverse primers (Table 1) were selected using the software Primer3 ⁵. After successful amplification of DNA fragments of 175 bp length, the essential conditions for the generation of KRAS fragments (157 bp) according to previously published method were met ⁶. As positive control samples, cancer cell lines (CCL228, CCL225) were processed in analogy to the historic tissue samples.

The tissue was surprisingly well conserved (age: 79-194 years old specimen; e.g. Figure 1). Histological diagnosis of the sections from the exhibited specimens was well applicable that allowed a definitive diagnosis in most cases. All specimens (12 pancreatic and 19 non-pancreatic tissues/tumours) were diagnosed according modern nomenclature as displayed in table 2.

In order to collect the required information about gDNA degradation, a multiplex PCR assay was developed based on the β -actin gene. A panel of four different fragments was therefore chosen as a representative sample including 120 bp, 175 bp, 365 bp, and 593 bp. After a first round of 35 PCR cycles, however, it became clear that the quality of isolated gDNA was not sufficient for direct amplification of the target fragments, despite some positive results. Notwithstanding the abundant generation of primer dimers in this first PCR round, the relevant region > 100 bp was not showing any unspecific side-products (Figure 2A). As a consequence, a second PCR round was estimated to be feasible and was performed using diluted PCR samples from the first cycling (dilution 1:10 - 1:100 in DEPC-treated water). This strategy yielded usually very strong signals at 120 bp (97% of samples positive) and 175 bp (72% of samples positive) thus paving the way for the RFLP analysis of KRAS (Figure 2B). In contrast, the higher fragments of 365 bp and 593 bp could be amplified only sporadically even after a total of 70 PCR cycles thus indicating fragments between 150 bp and 200 bp as maximum reliable template length for amplification. The relationship between amplified fragment length and positive PCR reaction is illustrated in figure 3.

Once successfully amplified, the KRAS fragment including codons 12 and 13 were digested with BStN I or Hph I in order to substantiate mutations on these codons. Strong PCR signals allowing for RFLP analysis were achieved in 22 out of 31 historic specimens reflecting a success rate of 71%. Similar to the PCR conditions of β -actin, a second round of 35 PCR cycles was necessary to generate the required amount of

PCR fragment suitable for RFLP (Figure 4). Among these 22 specimens, four mutations at codon 12 were detected by using agarose gel electrophoresis with DNA staining to visualize the digestion products (Figure 5). None of the investigated samples showed a mutation at codon 13. The four KRAS positive tumours were diagnosed as follows: adenocarcinoma of the trachea, a mature cystic teratoma of the ovary, a cortical carcinoma of the adrenal gland, and a glioblastoma multiforme. These results prove the feasibility of the presented method to detect KRAS mutations, but probably other genetic defects too, in historic specimen over 100 years old and conserved under very unfavourable conditions.

In conclusion, investigations with techniques of molecular pathology are a suitable approach to detect somatic mutations in historic formalin fixed specimens. To perform these tests, already known methodology of molecular pathology was fine tuned resulting in a complex work-up and amplification procedure including optimised gDNA isolation and two-stage multiplex PCR.

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We are deeply indebted to Mrs. Anica Krmpotic who processed and prepared paraffin embedded reference cells as positive control samples. This work was supported by the Hochschuljubiläumsstiftung der Stadt Wien, a research initiative of the city of Vienna.

For Peer Review

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Tables

Table 1: PCR-Primer for amplification of the β -Actin fragments (quality control of the isolated gDNA)

Primer	sequence	position*	length	PCR-fragment
Actin10/P	aaaatctggcaccacaccttcta	1474	23	120 bp
Actin14/M	actcacctgggtcatcttctcg	1593	22	
Actin8/P	gcatccacgaaactaccttcaac	2578	23	175 bp
Actin9/M	acaccaccttgatcttcattgt	2752	23	
Actin10/P	aaaatctggcaccacaccttcta	1474	23	365 bp
Actin11/M	ttgtcacacgagccagtgttagt	1838	23	
Actin12/P	gttgcgttacacccttcttgac	3016	23	593 bp
Actin13/M	cacacttggcctcattttaagg	3608	23	

* Position is related to the gen of β -actin (GenBank Acc# M10277)

Table 2: Historical cases with a comparison of the old and the modern diagnosis

#	Sex/Age	Year	Original Diagnosis	Revised Diagnosis
1	nk	1816	Atrophia pancreatis	Complete fibrotic atrophy
2	m/45	1819	Cystis capitis pancreatis	Chronic pancreatitis with pseudocyst
3 ^f	nk	1828	Cystides pancreatis multiplices	Chronic pancreatitis with pseudocysts
4	m/47	1831	Ductus Wirsungianus hydrope distensus	Obstructive chronic pancreatitis with dilation of the main pancreatic duct
5+	f/21	1834	Cystides pancreatis	Congenital cysts
6+	f/76	1840	Hydrops ductus Wirsungiani	Ductal adenocarcinoma pancreas (G2)
7+	f/64	1840	Cystadenoma pancreatis	Ductal adenocarcinoma pancreas (G1)
8+	f/24	1859	Carcinoma scirrhosum capitis pancreatis	Solid-pseudopapillary carcinoma
9+	nk	1862	Necrosis pancr. cum sequestratione	Necrosis of the pancreas with sequestration
10	f/45	1876	Carcinoma solidum capitis pancreatis subsequente hydrope ductus Wirsungiani	Neuroendocrine carcinoma
11	f/63	1882	Cystadenoma caudae pancreatis	Microcystic serous cystadenoma
12	f/46	ns*	Adenoma insulare pancreatis	Neuroendocrine tumor (insulinoma)
13	m/nk	1887	Carcinoma pharyngis	SCC pharynx G2
14	nk	ns*	Carcinoma papillare ves. felleae	Adenocarcinoma gall bladder G2
15	nk	ns*	Carcinoma metastaticum gland. suprarenalis Npl. bronchii	Metastasis neuroendocrine carcinoma
16	f/nk	1907	Carcinoma uteri	Endometrioid adenocarcinoma G1
17	f/nk	1907	Carcinoma uteri	SCC of the cervix G2

18	m/50	1914	Carcinoma vesicae urinariae	Neuroendocrine carcinoma G1
19	f/nk	ns*	Carcinoma ovarii	Serous cystadenocarcinoma
20	f/nk	ns*	Krukenberg-Tumour	Metastasis mucocellular carcinoma
21	f/nk	ns*	Carcinoma exculcerata mammae	Ductal adenocarcinoma breast G2
22	nk	ns*	Carcinoma tracheae	SCC trachea G1
23	nk	1926	Teratoma ovarii	Mature cystic teratoma
24	nk	ns*	Tumor medullae gland. supraren.	Phaeochromocytoma
25	m/21	1924	Myxoma verum atrii cordis	Cardiac myxoma
26	m/32	1931	Myxoma verum atrii cordis	Cardiac myxoma
27	nk	ns*	Tumor testis	Seminoma
28	m/nk	1924	Hypernephroma Grawitz	Clear renal cell carcinoma
29	m/66	1926	Hypernephroma Grawitz	Papillary renal cell carcinoma
30	nk	ns*	Adrenal cortical adenoma	Adrenocortical carcinoma
31	nk	ns*	Glioblastoma multiforme	Glioblastoma multiforme

Legend: f...female, m...male, nk...not known, ^f specimen collected by Johann

Wagner, +specimens collected by Carl Rokitansky, ns* not further specified: age between 1880-1920. SCC... Squamous cell carcinoma

Figures

Figure 1. Breast carcinoma of the 19th century grossly and histologically.

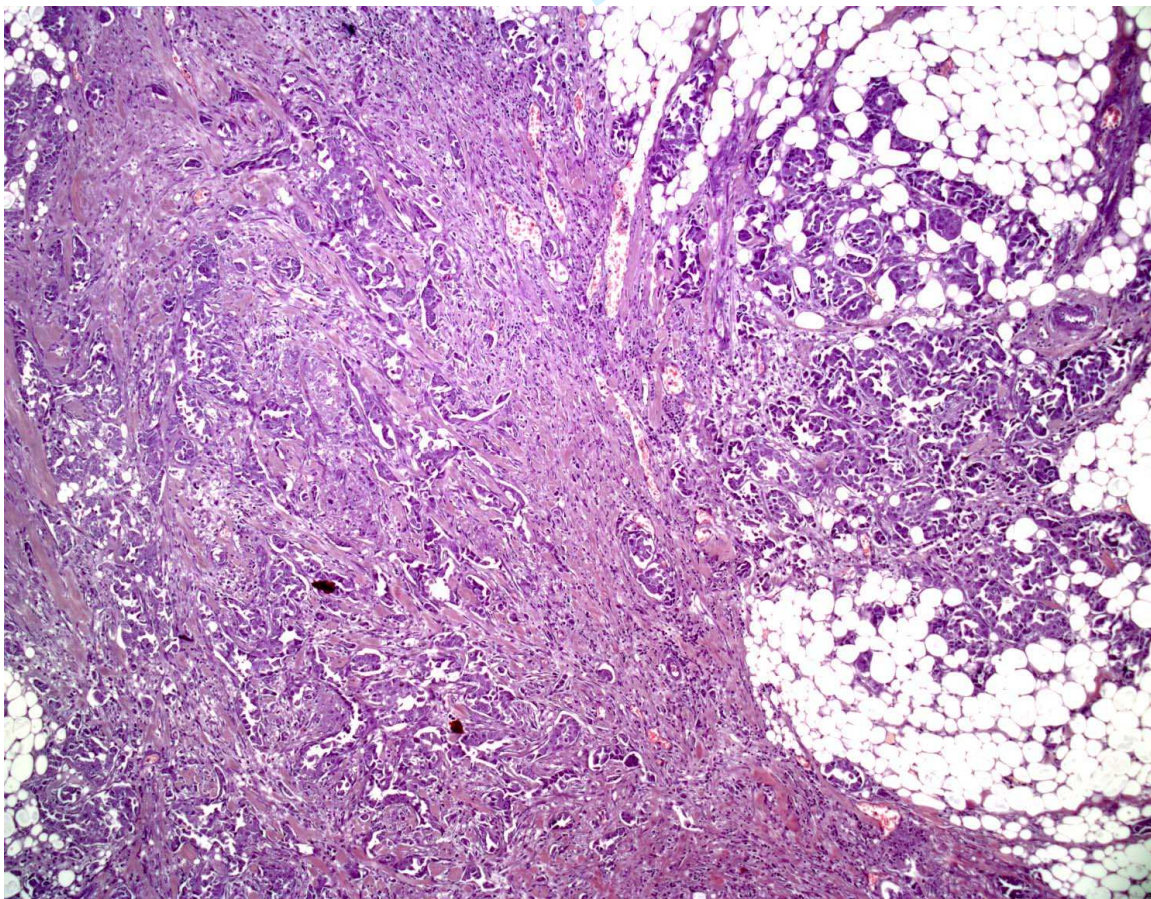
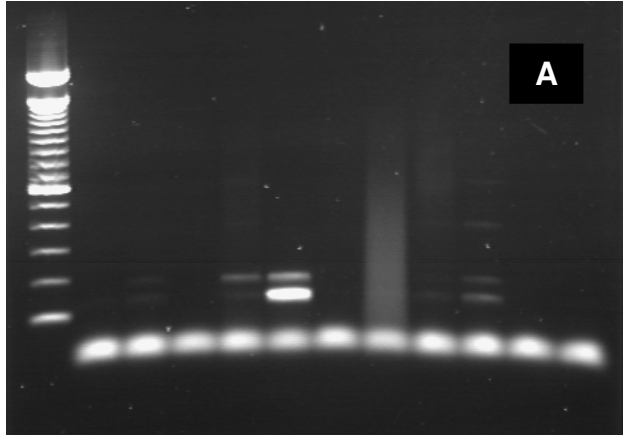
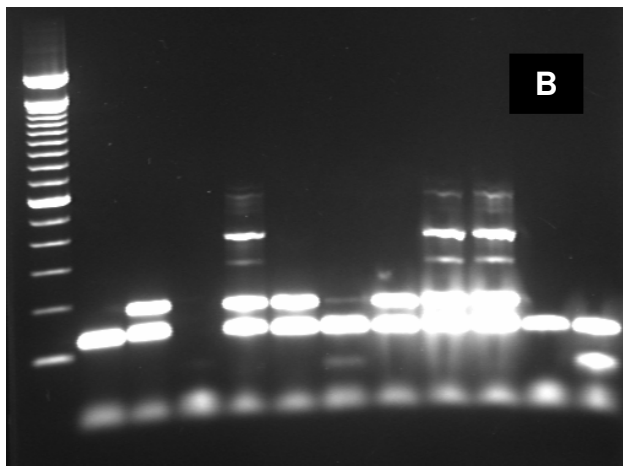


Figure 2. Multiplex PCR of β -actin gDNA isolated from historic specimen

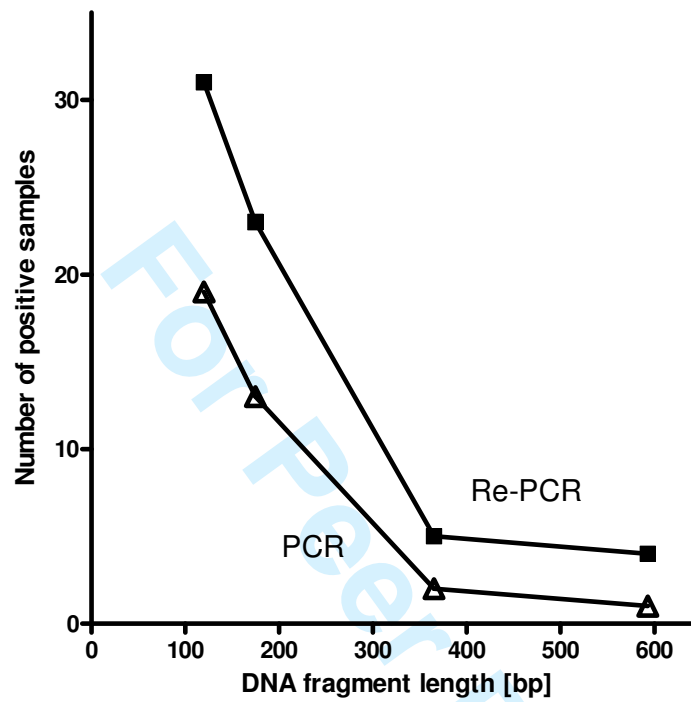


Legend: the signals visible after 35 PCR cycles on the agarose gel are mostly primer dimers with a few exceptions of amplified β -actin fragments. Lane M: 100 bp DNA marker; lanes 2 - 12: tissue specimen.



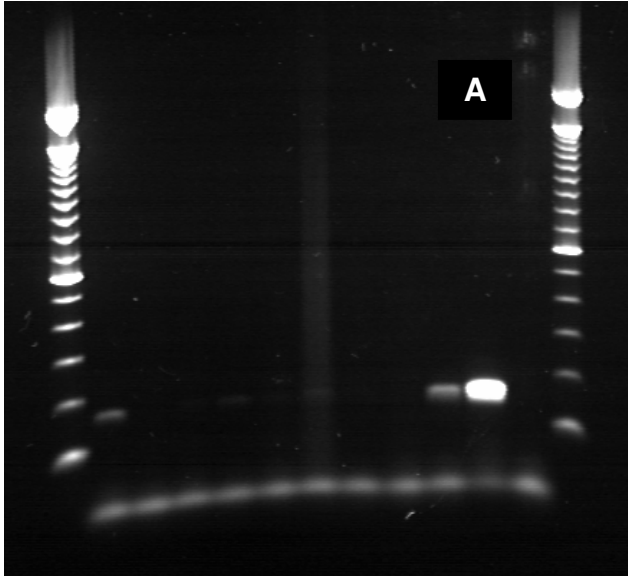
Legend: the signals visible on the agarose gel after re-PCR (70 cycles) are mostly shorter β -actin fragments (120 bp and 175 bp length), whereas primer dimers are considerably reduced. Lane M: 100 bp DNA marker; lanes 2 - 12: tissue specimen.

Figure 3. PCR amplification yield and DNA fragment length after 35 and 70 PCR cycles (gene: β -Actin)

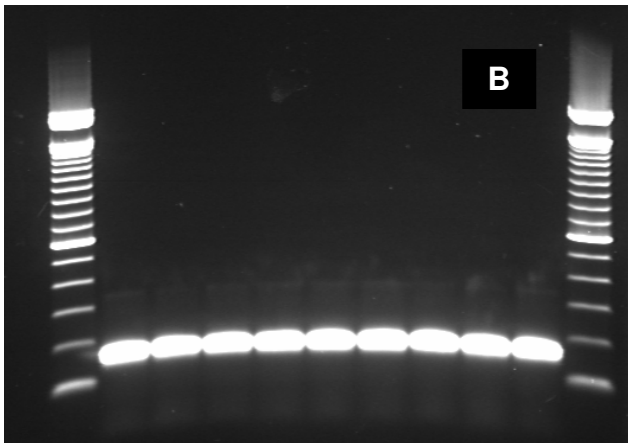


Legend: PCR refers to 35 PCR cycles, whereas signals from Re-PCR were generated by a total of 70 cycles

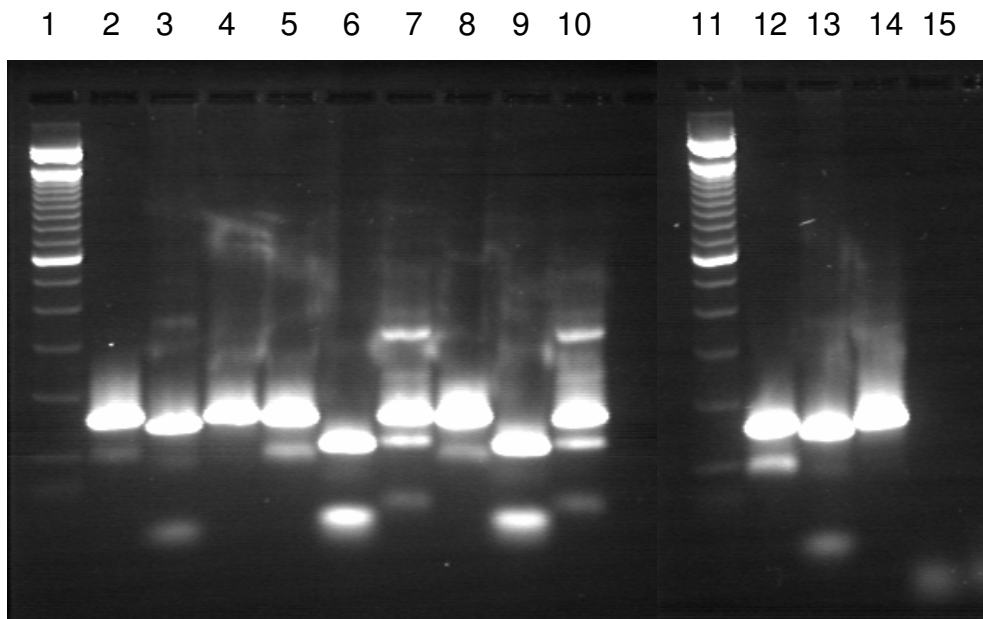
Figure 4. Amplification of exon 1 of the KRAS gene harbouring the frequently observed point mutations in codons 12 and 13.



Legend: the signals visible after 35 PCR cycles on the agarose gel are mostly primer dimers with a few exceptions of amplified KRAS fragments. Lane M: 100 bp DNA marker; lanes 2 - 12: tissue specimen.



Legend: the signals visible on the agarose gel after re-PCR (70 cycles) are KRAS fragments (157 bp length), whereas primer dimers are absent. Lane M: 100 bp DNA marker; lanes 2 - 10: tissue specimen

Figure 5. RFLP of KRAS point mutations on codons 12 and 13

Legend: Lanes 1 and 11: 100 bp DNA ladder; lanes 2 - 4: mutation detected in sample MN 30036 on codon 12 (lane 2: untreated reference sample with 157 bp fragment of the KRAS gene; lane 3: digest with BSt N I - 143 bp and 14 bp fragments - indicative for a mutation on codon 12; lane 4: digest with Hph I); lanes 5 - 7: mutation of codon 13 assessed in naive, positive control cell line CCL225 (lane 5: untreated reference sample with 157 bp fragment of the KRAS gene; lane 6: digest with BSt N I; lane 7: digest with Hph I - 157 bp, 114 bp, and 43 bp - indicative for LOH on codon 13); lanes 8 - 10: mutation of codon 13 in positive control cell line CCL225 embedded in paraffin (lane 8: untreated reference sample with 157 bp fragment of the KRAS gene; lane 9: digest with BSt N I; lane 10: digest with Hph I - 157 bp, 114 bp, and 43 bp - indicative for LOH on codon 13); lanes 12 - 14: mutation of codon 12 in positive control cell line CCL228 (lane 12: untreated reference sample with 157 bp fragment of the KRAS gene; lane 13: digest with BSt N I - 143 bp and 14 bp fragments - indicative for a mutation on codon 12; lane 14: digest with Hph I); lane 15: negative control