MEDICINA NEI SECOLI ARTE E SCIENZA, 18/3 (2006) 943-265
Journal of History of Medicine

Comunicazioni/*Posters*

MOLECULAR ANALYSIS OF A COLORECTAL CARCINOMA FROM A MUMMY OF THE XVTH CENTURY

MARIO FALCHETTI, RAMONA LUPI, LAURA OTTINI

Department of Experimental Medicine,
University of Rome "La Sapienza", Roma, I.

Introduction

Colorectal cancer (CRC) is one of the most frequent neoplasia nowadays. At molecular level, CRC results from the progressive accumulation of genetic and epigenetic alterations that lead to the transformation of normal colonic epithelial cells to colon adenocarcinoma cells. Loss of genomic instability appears to be a key molecular and pathogenetic step in the tumorigenesis process.

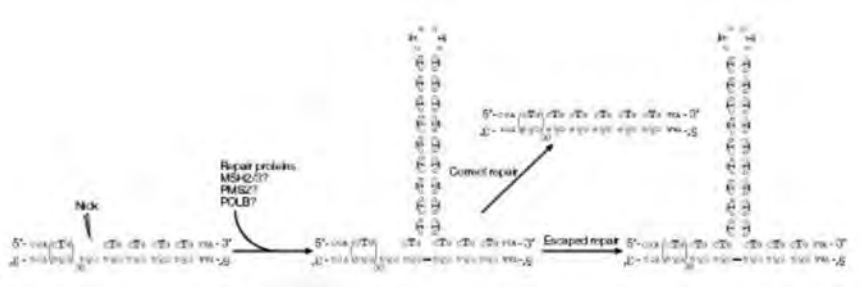
In the past tumors might have been rare. Molecular studies of tumors identified in ancient mummies may be helpful to shed light on the history of neoplasm and on the relationship between genetic alterations, lifestyle and environmental risk factors through time[1].

In the present study we investigated the molecular signatures, nowadays known to be related to CRC, in an adenocarcinoma infiltrating the small pelvis, identified in the artificial mummy of Ferrante I of Aragon, king of Naples in the second half of the XVth century.

Microsatellite instability

Microsatellite instability (MSI) is a form of genomic instability identified in CRC. MSI is characterized by high frequency of mutations in simple repetitive sequences. About 15% of sporadic CRCs have MSI[2]. Microsatellites are short repetitive sequences of

1 to 4 base pair. Because of their repetitive structure, microsatellite are prone to single-base mismatches that result in expansions/contractions in the number of the repeats, if the mismatch is not repaired. MSI is due to deficit in the mismatch-repair complex and is easily identified in tumors by analysing microsatellite loci in matched tumor and normal DNA. The microsatellite loci BAT25 and BAT26 are considered the most sensitive loci to identify MSI.



K-Ras

Point mutations in the oncogene K-Ras are the most frequent genetic alteration found in CRC, about 50% of all CRCs have K-Ras mutations[3].

Base substitutions at the K-Ras codons 12, 13 and 61 seem to be related to exposure to polycyclic aromatic hydrocarbons, the most important group of carcinogens deriving from the combustion of organic matter.

K-Ras is involved in cell growth regulation. Codon 12 (GGT) is the major mutational hotspot in CRC and shows a high susceptibility to damage by carcinogens generating bulky adducts on DNA.

↯

…GGA GCT **GGT** GGC GTA …

↓

constitutively K-Ras protein activation

BRAF

Point mutations in the BRAF gene are found in MSI-positive and in K-ras negative CRCs[3].

BRAF alterations may contribute to CRC carcinogenesis by up-regulating the anti-apoptotic role of the RAS/RAF/ERK pathway. A V599E hotspot mutation within the BRAF gene is present in about 45% of CRCs.

BRAF is a serin/treonin kinase involved in Ras signal transduction pathway. Ras stimulates Raf activation. V599E represents ≈90% of all BRAF mutations.

V599E causes500 fold increased protein activity

↓

constitutive Erk signaling pathway stimulation

Aims of the study

- To evaluate the MSI status and the presence of K-Ras and BRAF mutations in the adenocarcinoma found in the mummy of king Ferrante I of Aragon.
- To study possible associations between genetic alterations and environmental factors in the context of the XVth century .

Materials and methods

Samples.

Mummified tissues and formalin-fixed paraffin-embedded specimens, provided by Prof. Fornaciari, were available for the study. Manipulations of ancient samples, including DNA extraction, were performed in a laboratory located in a different building from the one where the PCR amplifications and sequencing were performed[4].

Ancient DNA extraction.

DNA was extracted from normal (muscle) and tumor samples.

Mummified tissue fragments were hand-homogenized and incubated in Proteinase K solution at 37°C O/N.

Formalin-fixed paraffin-embedded sections were dipped into xilene to remove paraffin, rehydrated in ethanol scale and incubated in Proteinase K solution at 37°C O/N.

DNA extraction from tissue pellets was performed using the GENECLEAN kit for Ancient DNA (Bio101, USA) according to manifacturer instructions.

Extreme precautions to avoid contamination were used to perform DNA amplification and multiple negative controls were always included[4].

PCR amplification.

Two-step PCR strategy was applied. Primers were specifically designed to amplify fragments of 110 bp for K-Ras exon 1, of 107 bp for BRAF exon 15 and of 125 bp for the Bat25 microsatellite locus. PCR were carried out using DNA samples from independent extractions.

PCR reactions were carried out in 50 ml containing 1 ml of pre-heated DNA at 50°C, 2.5 mM MgCl2, 0.2 mM each dNTP, 10 mM each primer, 1 U of Platinum Taq DNA Polymerase (Invitrogen) 20 mM Tris-HCl (pH 8.4), 50 mM KCl. PCR protocols consisted of an initial denaturation step at 94°C for 5 min followed by 40 cycles (94°C for 30 sec, 55°C for 30 sec, 72°C for 30 sec) in a thermal cycler (Perkin-Elmer 9600). The cycles were followed by final extension step at 72°C for 7 min.

For the second step of PCR, reaction was carried out under the same condition except that only 20 cycles were performed.

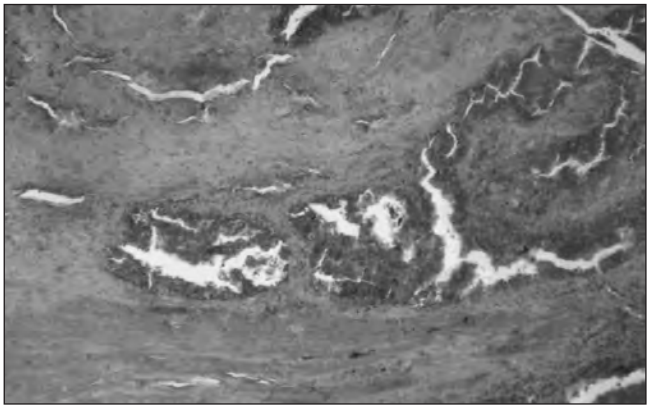
MSI analysis was conducted by using paired normal and tumor DNAs in a radiolabeled PCR assays.

All results were repeated three times.

Sarcophagi of San Domenico Maggiore Abbey in Naples and histologic section of tumor identified in the mummy of Ferrante I of Aragon [5,6]

Wooden sarcophagi of San Domenico Maggiore Abbey with the mummies fo Aragonese kings (upper) and Neapolitan nobles (lower)

King Ferrante I of Aragon Sarcophagus

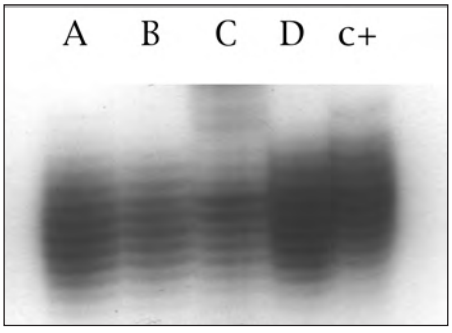


Adenocarcinoma infiltrating the small pelvis identified in the artificial mummy of king Ferrante I

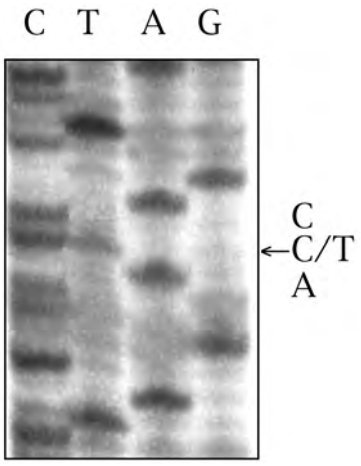
Results

MSI analysis at BAT25 locus
No alterations at the microsatellite locus BAT 25 were observed in tumor compared with normal DNA.

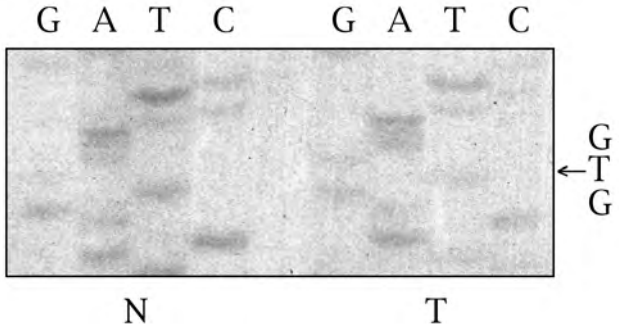
A : normal DNA;
B, C, D : tumor DNA from three independent extractions;
c+: control DNA.



K-Ras exon 1 sequencing analysis
The K-Ras codon 12 mutation (GGT>GAT) has been confirmed by direct sequencing in the tumor DNA of king Ferrante I of Aragon. Previous study conducted by using dot-blot analysis indicated the presence of codon 12 K-Ras mutation in tumor DNA[6].



BRAF exon 15 sequencing analysis
The BRAF V599E (GTG>GAG) mutation has not been detected in the tumor DNA from Ferrante I of Aragon by direct sequencing analysis. N: normal DNA; T: tumor DNA



Conclusions

The molecular analysis of the adenocarcinoma found in the mummy of king Ferrante I of Aragon, revealed the presence of K-Ras codon 12 GGT>GAT point mutation and the absence of MSI and of BRAF V599E mutation.

These findings are consistent with recent data showing that in CRC K-Ras and BRAF mutations are mutually exclusive. Moreover, BRAF V599E mutation was found to be associated with the presence of MSI. Indeed, CRC from Ferrante I of Aragon did not show MSI.

Interestingly, the K-Ras mutation identified has been associated with chemical carcinogens including polycyclic aromatic hydrocarbons and heterocyclic amines (HAs).

HAs interact with DNA to generate bulky adducts leading to mutations and they are generated in food grilled at a temperature of about 150°C, such as red meat, whose large consumption is considered a risk factors for CRC.

Intriguingly, as described in menus, dietary habits in the Aragounes court of Naples were characterized by large red meat intake and historical chronicles told about a strong consumption of grilled red meat by king Ferrante I of Aragon.

Acknowledgements

Study funded by MIUR-COFIN 2003 two-years research project: "Malattie e regime di vita nell'Italia centro-meridionale dei secoli XIII-XIX: fonti biologiche e storico-letterarie" coordinated by Prof. Luciana Rita Angeletti.

References

1. OTTINI L, LUPI R, FALCHETTI M, FORNACIARI G, MARIANI-COSTANTINI R, ANGELETTI LR. *Molecular paleopathology: a novel perspective for biomedical history*. Med Secoli. 2005;17(1):181-91.
2. SOREIDE K, JANSSEN EA, SOILAND H, KORNER H, BAAK JP. *Microsatellite instability in colorectal cancer*. Br J Surg. 2006 Apr;93(4):395-406.
3. OLIVEIRA C, VELHO S, MOUTINHO C, FERREIRA A, PRETO A, DOMINGO E, CAPELINHA AF, DUVAL A, HAMELIN R, MACHADO JC, SCHWARTZ S JR, CARNEIRO F, SERUCA R. *KRAS and BRAF oncogenic mutations in MSS colorectal carcinoma progression*. Oncogene. 2006, Sep 4.
4. POINAR H. N. *The top 10 list: criteria of authenticity for DNA from ancient and forensic samples*. Int Congress Series 1239 (2003) 575-579.
5. FORNACIARI G. *The mummies of the Abbey of Saint Domenico Maggiore in Naples*. Paleopathol Newsl. 1984 Sep;(47):10-4.
6. MARCHETTI A, PELLEGRINI S, BEVILACQUA G, FORNACIARI G. *K-RAS mutation in the tumour of Ferrante I of Aragon, King of Naples*. Lancet. 1996 May 4;347(9010):1272.