

LO SCREENING UNIVERSALE DELLA SINDROME DI LYNCH : IL MODELLO FELTRE

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Nell'ULSS1 Dolomiti (Veneto) opera dal 2012 un gruppo multispecialistico per la diagnosi e la gestione di pazienti con Sindrome di Lynch (SL), la più comune forma eredo-familiare di carcinoma coloretale. Dato che l'utilizzo dei soli criteri clinico-computazionali determina la perdita di una percentuale sostanziale di pazienti affetti, presso il nostro centro viene attuato uno screening universale esteso a tutti i carcinomi del colon-retto e dell'endometrio di nuova diagnosi con una consulenza genetica pre-test e l'esecuzione dei test di primo livello (IHC proteine MMR, Instabilità Microsatellitare, mutazione BRAF e metilazione del promotore MLH1). Su 170 casi sottoposti a screening, 23 pazienti sono stati inviati all'analisi molecolare di secondo livello. Di questi, 12 pazienti con MSI-H e prevalente perdita di espressione del dimero MLH1/PMS2, non hanno dimostrato mutazioni a carico dei geni del MMR ma solo varianti di sequenza non patogenetiche e classificate come sindromi Lynch-Like. Nei restanti 11 casi è stata individuata una mutazione a carico dei geni del MMR con una duplicazione ricorrente a carico del gene MSH2 a causa verosimilmente delle caratteristiche geografiche peculiari della nostra regione (comunità confinate). Lo studio mutazionale genetico esteso ai famigliari ha consentito di identificare 8 soggetti con mutazione dei geni del MMR su 16 inviti. In conclusione il lavoro condotto dimostra la validità dello screening universale come vero driver nella identificazione e diagnosi di soggetti portatori di SL.

Genetic counselling unit- model and experience of University Hospital for Tumours in Croatia

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Genetic counselling unit exists as a separate functional unit in University hospital for tumours from the year 2015. It is based on cooperation and teamwork of experts of different profiles: medical geneticist, surgeons, oncologists, molecular geneticists, specialized nurses and psychologists. The aim is to recognize and counsel patients with family and personal history indicative of the hereditary cancer syndrome. Most of the patients are those suspected to have *hereditary breast and ovarian cancer syndrome*, although there are also patients with familial adenomatous polyposis, Lynch syndrome (hereditary non-polyposis colorectal cancer syndrome), neurofibromatosis and few other very rare hereditary cancer syndromes (e.g. von Hippel-Lindau disease, Li Fraumeni).

Clinical signs pointing to the possibility of pathogenic mutation in some of the predisposing genes (early age of onset, typical histology, multiple tumours...), along with confirmed family history are the base of selection. Patients with such disease characteristics are referred to a genetic counselling centre with the assessment of indication for further genetic testing.

Genetic testing of *hereditary breast and ovarian cancer syndrome* (*BRCA1* and *BRCA2* genes), as the most commonly identified hereditary cancer, is now available and covered by Croatian health Insurance Fund through the programme of genetic counselling in an authorized institution. The analysis is performed by next generation sequencing technology coupled with quantitative polymerase chain reaction (qPCR) for detection of large deletions and duplications. The positive result is confirmed by Sanger sequencing. Genetic testing is performed in a Laboratory for hereditary cancer and Laboratory for advanced genomics at Rudjer Boskovic Institute, Zagreb. Patient receives genetic counselling before and after genetic testing.

Primary and secondary prevention measures that could be undertaken after the results of hereditary breast cancer genetic testing in a high risk population contribute to savings in the health care system and the better quality of life in a group of high risk individuals.

Keywords: genetic counselling, genetic testing, hereditary cancer syndromes, Croatia

Universal screening to identify Lynch Syndrome: two years of experience.

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Recently different platforms have been tested for LS universal testing including germline molecular analysis, somatic microsatellite instability (MSI), immunohistochemical analysis (IHC) on CRC. The most cost effective methods include IHC and or MSI however the effectiveness of the strategy depends on the healthy policy of each country.

According to these findings, Lombardy Region in 2015 improved LS screening network (Delibera n°4498 3/6/2015) recommending to include MMR IHC expression in all diagnostic reports of resected CRC as universal screening and to suggest Cancer Genetic Counselling for all patients affected by a MMR defective CRC.

Here We reported two years of experience of IHC universal screening to identify LS patients in Ospedale di Circolo, ASST Settelaghi in Varese (Italy).

A cohort of 352 consecutive cases of surgical CRC diagnosed from 1st September 2015 to 31st August 2017 from 346 patients (6 patients had two CRCs) was routinely evaluated for MSH2, MLH1, MSH6 and PMS2 protein expression using immunohistochemical approach.

A MMR defect was identified in 70 out of 352 CRCs (19.9%) from 68 patients. Among the 61 MLH1 immunonegative tumors, MLH1 promoter hypermethylation was detected in 56 (92%) cases and V600E BRAF mutation was found in 41 out of 61 (67%) CRC.

Cancer Genetic Counselling was offered to all 68 patients affected by MMR defective CRC and 25 patients offered to this service (compliance of 40%).

Using combined somatic approaches in order to ascertain MMR defect we identified 2 out of 25 (8%) LS patients and also two cases suspected for Lynch Like syndrome.

Our results suggest that universal screening including BRAF V600E and MLH1 methylation analyses is an efficient approach to identify high risk patients for CRC especially in a cohort of old aged patients.

Streamlining Universal Screening for Lynch Syndrome (LS): towards improved yield of Genetic Counseling (GC)

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BACKGROUND. LS is largely underdiagnosed although Universal Screening (US) in colorectal cancer (CRC) patients through Mismatch Repair deficiency (MMR-d) testing is widely endorsed. Low adherence to guidelines among oncologists may be partly due to a lack of consensus on whether all MMR-d patients should be referred to GC/Genetic Testing (GT). As *BRAF* mutation rules out LS, we estimated the increased yield of LS diagnosis from GC /GT which could be obtained by selecting candidates for GC through *BRAF* testing.

METHODS. From 2011 to 2016, 1447 consecutive stage I-IV CRC surgical patients at a single institution, underwent immunohistochemistry (IHC) for LS using anti MLH1, MSH2, MSH6 and PMS2 antibodies. Oncologists were invited to refer all MMR-d patients to GC/GT. *BRAFV600E* testing was carried out only in case of MLH1 protein loss at IHC.

RESULTS. MMR-d was found in 194 patients (13%), with 171 showing loss of MLH1 expression (88%). Oncologists referred 27 (16%) to GC. Among the 21 who underwent GC, *BRAF* testing and GT, 9 were *BRAF* wild type (wt) (43%) and none had LS. Among the 23 MMR-d patients with loss of expression of MSH2, MSH6 or PMS2 (\neq MLH1), oncologists referred 9 to GC (39%): 7 underwent GC / GT and 3 carried LS (43%) at GT. Median age was 76 years (range 30-97) in the MMR-d group, 78 (range 41-97) in the MLH1 group and 63 (range 30-86) in the \neq MLH1 group. Overall, LS was diagnosed in 3 of the 28 MMR-d patients (11%) who underwent GC /GT, possibly an underestimate due to the advanced median age of our MLH1 loss patients. Had we only offered GC to the 9 *BRAF* wt patients among the 21 with MLH1 loss, we could have avoided 12 (57%) of the GC sessions conducted, increasing the yield of LS diagnosis from 3/28 (11%) to 3/16 (19%) (75% increase).

CONCLUSIONS. When US for LS is adopted, a GC referral rate reduction of 57% among MLH1 loss patients, and an overall increase in the yield of GC of about 75% can be obtained by testing for *BRAF* mutation before oncologist referral to GC rather than after. As multistep selection of patients by oncologists may be unfeasible, CRC pathology reports with combined MMR-d and *BRAF* testing (for MLH1 loss at IHC) and an 'LS suspicion alert' could improve oncologists' awareness of LS and compliance with guidelines.

The role of immunohistochemistry (IHC) testing in Lynch Syndrome (LS) cancer spectrum

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Aim To validate the performance of IHC testing of mismatch repair (MMR) proteins in patients with LS spectrum cancers.

Methods We analyzed microsatellite instability (MSI) and IHC of MMR proteins in 541 cancers: 446 colorectal (CRCs), 37 endometrial (ECs), 31 ovarian (OCs), 27 other sites. Samples were classified as MSI-High, MSI-Low or microsatellite stable (MSI-H, MSI-L, MSS). IHC results were proficient-IHC (normal expression), deficient-IHC (loss of expression), borderline-IHC (“patchy” expression). Excluding samples with *BRAF* p.Val600Glu mutation or *MLH1* promoter hypermethylation (*MLH1*-Hy), deficient-IHC cases were sent to genetic counseling.

Results We identified 471 proficient-IHC, 51 deficient-IHC, 11 borderline-IHC, 8 inadequate samples; 8 borderline-IHC tumors were MSI-H and then classified as deficient-IHC cases.

Overall, 59 samples had deficient-IHC: 1 for all MMR proteins, 1 for 3 proteins, 46 for 2 proteins (36 *MLH1*-PMS2, 10 *MSH2*-*MSH6*), 11 for 1 protein. IHC deficiency rate was different among sites: 9% CRCs, 35% ECs, 13% OCs, 0% other sites ($p < 0.0001$).

Thirty-one samples had *BRAF* mutation or *MLH1*-Hy. The remaining samples were from 26 patients: 12 have already been tested and 10 germline variants were detected (4 in *MLH1*, 4 in *MSH2* and 2 in *MSH6*, including 1 borderline-IHC with MSI-H).

Conclusions We support the systematic evaluation of all MMR proteins in CRCs and gynecological cancers to select patients with LS. MSI appears useful to manage borderline-IHC cases.

***EPCAM* deletions in Lynch Syndrome: report of new variants in Italy and the associated molecular phenotype**

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MSH2 promoter methylation as a consequence of a constitutional deletion of the upstream *EPCAM* gene is found in about 1-3% of Lynch Syndrome (LS) patients and represents a classical secondary epimutation, heritable and tissue-specific.

In 224 LS patients with a pathogenic variant, we have found an *EPCAM* deletion in 5 unrelated cases (about 2%), consistent with previously estimated frequencies. All carriers were affected by CRC at young age (25-50 yrs) –often multiple, MSI-H and negative *MSH2/MSH6* IHC- and had a positive CRC family history. Among the 20 relatives tested, 8 healthy and 3 CRC-affected subjects were positive for the proband's deletion. Molecular characterization evidenced that all breakpoints involve two *Alu* regions with very high homology. *EPCAM* variants *c.556-490_*8438del* (shared by two families) and *c.858+1193_*5826del* are novel; *c.859-1430_*2033del* and *c.859-670_*530del* have been yet reported in European families.

Both normal and tumor colon tissues displayed concomitant *MSH2* promoter methylation, acting as a first-hit for gene inactivation. In two cases MLPA showed a large somatic deletion including also the *MSH2* gene, causing its complete functional loss in tumor cells. Together with out-of-frame *EPCAM/MSH2* transcripts, we also had evidence of in-frame fusion transcripts. These latter are predicted to be translated into fusion proteins, possibly with aberrant location, as evidenced in two CRC samples showing cytoplasmic *MSH2* staining.

Along with a total of 16 CRCs, the tumor spectrum of carriers included 1 pancreas, 1 duodenum and, according to previous genotype-phenotype correlations, endometrial cancer was absent.

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Whole Exome Sequencing in pazienti con poliposi amartomatose non spiegate

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In un ventenne con diagnosi clinica di Sindrome Peutz-Jeghers (melanosi labiale, invaginazione ileale a 12 anni; asportazione di polipi di Peutz-Jeghers del tenue a 15 anni), senza varianti identificate in STK11, il Whole Exome Sequencing (WES) ha evidenziato due varianti missenso in eterozigosi predette dannose: P456T nel gene APC2 e K830R nel gene RICTOR. Le varianti, tuttavia, sono risultate ereditate dalla madre, 49 anni, asintomatica (se si eccettua lentiginosi periorale); inoltre, un polipo di Peutz-Jeghers asportato al paziente mostrava persistenza di eterozigosi per la variante di APC2 e perdita dell'allele mutato di RICTOR.

La seconda paziente, 25 anni, con ritardo mentale lieve, anomalie EEG e dolore addominale cronico, è stata sottoposta dall'età di 11 anni a rimozione di polipi multipli con diversa istologia: polipi giovanili del colon e dell'ano, polipi iperplastici multipli e polipo ganglio-neuromatoso del colon. Analisi mutazionale di PTEN, BMPR1A e SMAD4 negativa. L'analisi WES ha evidenziato le varianti potenzialmente dannose L86P di STK11I e P1143S di DUOX1. Esse, però, sono risultate ereditate dalla madre e presenti anche in una sorella, entrambe asintomatiche.

In conclusione, il sequenziamento esomico non è risultato efficace per identificare le cause genetiche di poliposi amartomatose non spiegate, il che supporta l'ipotesi che il difetto genetico in casi come questi risieda in regioni regolatorie o introniche dei geni noti, piuttosto che in nuovi geni.

LINE-1 activity and metabolic switch evaluation in *MUTYH*-associated polyposis model

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Accumulation of G>T transversion at specific gene loci and DNA global demethylation are proper of *MUTYH* associated polyposis (MAP), a hereditary syndrome characterized by unrepaired oxidative DNA damage. Long Interspersed Nuclear Element-1 (L1), which are repeated sequences activated in cancer through DNA demethylation, can be partially regulated by Sirtuin 6 (Sirt6), an oxidative stress resistance deacetylase involved in the anaerobic switch of colonocytes. We hypothesized a crosstalk between L1 activation and genetic and/or metabolic alterations in the MAP model. To this purpose, the L1-coding gene (*ORF1/2*) expression was compared with the *SIRT6*, *LDH-A*, *PDK1* and *PDK3* metabolic -associated gene transcript level and/or the presence of MAPK gene mutations in MAP adenomas, using qPCR and mass-spectrometry. Sporadic adenomas and normal colic mucosa were used as controls. As expected, MAP adenomas showed an accumulation of *KRAS* G12C mutation, with few of these lesions exhibiting multiple MAPK gene mutations. Overall, MAP adenomas displayed higher L1 gene expression levels compared to normal mucosa. As for the metabolic controlling genes, MAP polyps were characterized by higher *LDHA* mRNA expression but lower *SIRT6* transcript level compared with normal mucosa (p-value ≤ 0.0001). Notably, the higher expression of L1 genes was found in MAP wild-type adenomas, whereas MAP mutated polyps displayed an increased *SIRT6* expression. According to these results, the onset of specific *KRAS* mutations and the activation of L1 gene expression, with the concomitant loss of *SIRT6*, can independently support the oxidative damage-associated colorectal carcinogenesis. Both these events seem to be associated with a very early metabolic switch.

Generation of 3D Organoid cultures from FAP Patients harboring *APC* and *MUTYH* mutations

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Familial Adenomatous Polyposis (FAP) is an autosomal dominant condition caused by germline inactivating mutations in the Adenomatous Polyposis Coli (*APC*) and mutY homologue (*MUTYH*) genes, characterized by presence of hundreds to thousands of polyps throughout the colorectal mucosa. *APC* mutations give theoretically 100% risk of developing a CRC; *MUTYH*-associated polyposis is characterized by attenuated polyposis. In these individuals, prophylactic surgery is recommended in order to prevent CRC development.

Three Dimensional Patient Derived Organoids (3D-PDO), obtained by allowing cells to self-organize into a 3D structure that reproduces the original organization of the surgical specimen, recapitulate more closely than conventional cell lines the physiology of tumors and are an ideal model for conducting *in vitro* studies.

To better understand the mechanisms underlying the growth and development of polyps in FAP we have set up a procedure to generate 3D-PDOs from adenomas derived from colectomies of FAP patients. Specimens have been processed and cultured starting from the protocol developed by Fuji et al. (Cell Stem Cell 2016), with some modifications. Immunohistochemistry analyses have confirmed that organoids recapitulate the physiopathology of the tissues of origin. *APC* and *MUTYH*-derived organoids show different conditions of growth that resemble their clinical behavior, highlighting that 3D-PDOs are solid and reproducible models to be used for deepen the knowledge on FAP.

Multilocus Inherited Neoplasia Alleles Syndrome: a series of four cases

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The frequency of Multilocus Inherited Neoplasia Alleles Syndrome (MINAS) cases is probably underestimated because, particularly in the past, the standard approach was to test candidate inherited cancer genes sequentially until one pathogenic variant was identified. Although rare, such conditions should be suspected whenever the phenotype or the inheritance pattern suggest more than one underlying genetic cause. The increased use of multigene panels in routine clinical practice is expected to rise the identification of cases of MINAS.

Here, we report 4 new MINAS cases: two involve the combination of pathogenic variants in *MSH6* with *APC* and *TP53*, respectively, one in *MSH2* and *PTEN*, and the fourth in *APC* and *MUTYH*. To our knowledge, none of such combinations has been previously reported. Interestingly, each deleterious variants seem to have an independent effect and patients didn't show unusual or more severe phenotype.

These reports add to the growing body of literature of patients with MINAS identified thanks to careful personal and family history-taking combined with the increasing use of multigene panels. However, the cumulative cancer risks for patients who harbor deleterious variants in multiple inherited cancer genes is still poorly characterized. Collecting information on the phenotypic spectrum of MINAS cases is essential to better define what the effect of a particular combination of deleterious variants might be and to improve the clinical management in these patients.

Multiple-gene panel analysis in an Italian cohort of patients with familial gastric cancer

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The major gene involved in gastric cancer (GC) predisposition is *CDH1*, but other genes have recently emerged as possibly predisposing to the disease.

The aim of our study was to assess the presence of predisposing variants in Italian patients with GC family history by analyzing a panel of 94 genes involved in the main cancer syndromes.

We selected 79 patients with GC, 3 patients with gastric polyposis and 14 patients with lobular breast cancer showing strong family history of GC. Genomic DNA was extracted from peripheral blood and analyzed by Next-Generation Sequencing.

In 10 out of 96 patients (10.4%), we identified 9 *CDH1* pathogenic variants.

In 11 out of 96 patients (11.5%), we found 11 pathogenic variants in unexpected genes, including *ATM*, *BLM*, *BMPRIA*, *BRCA1*, *BRCA2*, *MSH2*, *PALB2*, *PMS2* and *PRF1*.

In 75 out of 96 cases (78.1%) we did not find any clear functional variant. The 75 patients showed 156 unique missense variants with a population frequency <1%. We evaluated them by using PolyPhen-2/SIFT bioinformatic tools and 27 variants were classified as probably damaging by both tools.

The majority of the pathogenic variants identified were in genes related to breast cancer (*BRCA1*, *BRCA2*, *ATM*, *PALB2*), while others were in genes involved in the susceptibility to colorectal cancer (*MSH2*, *PMS2*, *BMPRIA*) and to multiple cancers (*BLM*, *PRF1*). Further studies based on segregation analysis and functional assays will definitely confirm the role in GC development of the variants identified.

Possible attenuator effect of intronic STK11 variant in a Peutz-Jeghers family with germline pathogenic splicing mutation showing high phenotypic variability.

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Peutz-Jeghers Syndrome (PJS) is an autosomal dominant pre-cancerous disorder, caused by germline mutations in the tumor suppressor gene *STK11*, in about 80% of cases. We performed a genetic test of *STK11* in two Italian young sisters in which Peutz-Jeghers syndrome was suspected, since they showed pathognomonic *café au lait* spots in absence of other symptoms and familiarity. Sequencing of the 9 exons of *STK11* led to the identification, in both the probands, of a novel germline mutation named c.597G→A, hitting the last nucleotide of exon 4. Interestingly, genetic testing of the two probands' parents showed that the unaffected father was carrier of the mutation. Moreover, he carried an intronic substitution named c.465-51T→C which was not inherited by his daughters. We also observed that all subjects carrying the c.597G→A mutation presented an aberrant splice variant of *STK11* mRNA lacking exon 4. *In silico* analysis of c.465-51T→C substitution showed that it probably activates a silent Enhancer Splicing Element affecting mRNA splicing. Finally, qRT-PCR analysis of *STK11* expression levels showed a slight downregulation of wild type allele in the father and about a 2-fold downregulation in the probands compared to the mother. Our results gave rise to the hypothesis that the c.465-51T→C intronic variant, which segregates with the wild type allele, could compensate for the splicing mutation c.597G→A effect, being responsible for the phenotypic variability observed in this family.