Università degli Studi del Piemonte Orientale

Scuola di Medicina

Dottorato di Ricerca in Scienze e Biotecnologie Mediche (XXIX ciclo)

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Final PhD report for the first year

Defining the role of small DNA skin-tropic viruses in the development of skin cancer in the immunocompromized host through high-throughput approaches

Background

The growing population of immunosuppressed individuals (e.g. organ transplant recipients, OTRs) alongside primary immunodeficiency (PID) has created a group of patients with increased incidence of specific cancers, most of which are caused by reactivated viruses whose oncogenic potential is suppressed by the immunological reaction in healthy individuals. Skin cancer is the most frequent form of malignant cancer in these individuals. In addition to the other well known risk factors for skin cancer, numerous studies have also pointed to a possible causal role of small DNA tumour viruses in the pathogenesis of skin cancer, including Human *Betapapillomaviruses* (β-HPV) and Polyomaviruses (HPyV), although a causal role of these viruses has been difficult to verify using epidemiological approaches because of their ubiquitous prevalence in the general population (1,2).

This association has been clearly defined in patients suffering from epidermodysplasia verruciformis (EV), a primary immunodeficiency (PID) characterized by increased susceptibility to β-HPV genotypes (3,4). EV begins during infancy or childhood and is manifested by disseminated highly polymorphic skin lesions that carry a considerable risk of malignant transformation. HPV types belonging to genus *Beta* are consistently found in EV tumors and are regarded as causative agents (5). EV is thought to be an autosomal recessive disease; homozygous mutations in EVER1 or EVER2 have been identified in approximately 75% of patients given a diagnosis of EV, leaving a considerable proportion of patients with an unexplained genetic cause (6,7,8).

Another PID predisposing to HPV-associated disease is WHIM syndrome (Warts, Hypogammaglobulinemia, Infections and Myelokathexis). These patients have a specific, poorly understood susceptibility to α-HPV-induced warts, usually manifesting in the second decade of life. Condylomas in the genital region and genital cancers, always caused by α-HPV genotypes, have also been reported. WHIM syndrome is inherited in an autosomal dominant fashion and is caused primarily by heterozygous gain-of-function mutations in the gene encoding the chemokine receptor CXCR4 (9).

Another setting with increased HPV and HPyV susceptibility is represented by patients with iatrogenic immunodeficiency, such as that occurring in organ transplant recipients (OTRs). Cutaneous squamous cell carcinoma (cuSCC) is the most common malignancy in these patients with an approximately 100-fold increased incidence compared to the general population (10).

Because most of the studies so far performed in the humans were designed to evaluate the presence of and serological responses against known HPV and HPyV genotypes, they leave open the possibility that: i) undiscovered HPV or HPyV species or variants that are not detectable or identified with available detection assays may be more strongly associated with cancer; ii) they may

be only involved during the early stages of skin carcinogenesis and not necessarily maintained in more advanced stages, iii) co-infection and synergistic interactions may occur among viruses from the same or different viral family.

Objectives

Based on this premise, my research project has been aimed at:

i) performing viromic analysis of PID patients for virus discovery and characterization of disease-associated viral polymorphisms of known viral species;

ii) dissecting natural HPV and HPyV infection/reactivation (including new viruses) in immunosuppressed patients in order to establish their causative role (either independently or synergistically) in the conversion of keratinocytes towards malignancy;

During the first year of my PhD, I have been working on the following tasks:

i) Harvesting of biological materials (blood, skin swabs, hair bulbs, skin tumors) from naturally immunosuppressed individuals, including EV, WHIM and other unclassified PIDs attending the University-Hospital of Novara, and Spedali Civili, Brescia, and from organ transplant recipients (OTRs) attending the University-Hospital of Novara.

ii) Detection of HPV and HPyV-DNA in the skin swabs obtained from selected patients as described above. DNA was extracted by using the QIAamp Tissue Kit (Qiagen). The presence of different HPV and HPyV species was then determined by a PCR approach based on a degenerate primer set able to detect a broad spectrum of β-HPV (CP62-CP70a for the first amplification step and CP65-CP69a for the second amplification step), amplifying a 421 bp long region within the L1 ORF. Then, we have analized mucosal genotype α-HPV: (MYN primer, 370bp) (GP5/6 primer,140bp); and a series of skin-tropic polyomavirus (HPyV): MCPyV (qP1/qP2 primer), (lT1 primer, 432bp), (lT3 primer, 308 bp), (VP1 primer, 355 bp), (M1-M2 primer,178bp); HPyV6 (124bp); HPyV7 (227bp); TSPyV (sT primer,577 bp)(lT primer, 119 bp).

iii) Virions preparation and high-throughput deep sequencing. Skin swabs from a series of PIDs patients already characterized by our group, have been processed by sequential sedimentation and density gradient ultracentrifugation for virions purification. DNA has been extracted from gradient-purified virion preparations and subjected to random-primed rolling circle amplification (RCA) to further enrich for viral sequences. RCA is a powerful technique for the specific detection of circular DNA molecules, such as the circular DNA viral genomes of HPVs and HPyVs. In this year I have been able to prepare RCA samples from 3 WHIM patients, 2 EV, one patient with severe plantar warts, and one patient with Dock8 genetic defects. All these samples have been already sent to Alberto Peretti, a post doc from our laboratory currently working at the National Cancer Institute NIH (Bethesda-USA) in the laboratory of Chris Buck. Alberto is performing deep sequencing using Illumina Nextera XT sample preparation kit and the Illumina MiSeq platform. The results obtained will allow us to define the skin virome of these immunosuppressed patients and hopefully identify new viruses or variants of the known genotypes.

Moreover, I did molecular and histological analyses of the samples from a young healthy boy with a history of recalcitrant multiple large plantar warts that revealed high viral loads of a variant of the HPV2 α-genotype in both lesional and non-lesional skin sites. To gain more insight into the patient's skin viral microbiota, DNA extracted from skin swabs from the affected foot was rolling-circle-amplified and deep sequenced. Thirty percent of all reads could be assigned to HPV genomes. All sequences matching HPV genomes displayed best alignment with HPV2 genomes. Interestingly, more than 95% of the HPV2-related reads showed the presence of 7 nucleotide substitutions; including one point mutation at nucleotide 7720 in the LCR region and a novel amino acid exchange (V:A) in the E2 protein. The observed genomic variations may contribute to the unusual clinical manifestation of the infection. Since treatment with intravenous cidofovir was completely ineffective, HPV vaccination was decided and lesions significantly decreased in size and thickness, supporting the therapeutical use of the tetravalent vaccine in HPV2-induced recalcitrant warts (poster session at the 42° congresso nazionale della Società Italiana di Microbiologia).

iv) As described above, we are currently harvesting skin biopsies form immunosuppressed individuals, either OTR or PID, who underwent surgical treatment of skin cancers in the frame of a long lasting collaboration with the dermatology and nephrology units in the Medical School in Novara. Half of each biopsy is FFPE or snap-frozen, and their sections will be used for immunofluorescence and pathology evaluation. The other half is used for xenografting in nude mice. The *in vivo* orthotopic xenograft assay using a "humanized" stromal bed repopulated with human fibroblasts established a couple of years by Girish Patel is currently successfully employed in our laboratory to implant human skin SCC tissue for the routine growth of tumors. I have been deeply involved in the setting up of this technique and I have been able to implant 17 tumours during this first year, 8 of them grew in the nude mice, and xenografts were removed and are currently under evaluation.

Bibliography

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Meetings' participation

- 42° congresso nazionale della Società Italiana di Microbiologia.
  Torino, centro congressi Torino incontra
  From 9-28-2014 to 9-1-2014

- 3rd Workshop on Emerging Oncogenic Viruses.
  San Pietro in Bevagna, Manduria, Italy.
  From 6-4-2014 to 6-8-2014.

- 7th International Conference on HPV, Polyomavirus and UV in Skin Cancer, Novara, Italy.
  From 4-9-2014 To 4-12-2014

Poster :

SIM (società italiana di microbiologia) Torino, centro congressi 'Torino incontra' 28 settembre-1 ottobre 2014

Identification of the skin virome in a young boy with severe HPV2- positive warts that completely regressed after administration of tetravalent human papillomavirus vaccine.
Olivero C., Landini M.M., Peretti A., Matteelli A., Urbinati L., Mignone F., Lai A., Doorbar J, Borgogna C., Gariglio M., and De Andrea M.

SEMINARS 2014

Oral presentation of PhD student of XXVII CYCLE

-1/10/2014 14:00 Ciardullo Carmela "Clonal evolution andclinical relevance of subclonal mutations in chronic lymphocyticleukemia"

The Borghese Sessions Steven R Ellis

-September 8
10:00 Clinical case – Skin as an organ
11:00 Layers of skin, cell types, developmental origins

-September 9
10:00 Cell-Cell Interactions – anchoring junctions
11:00 Cell-Cell Interactions – occluding junctions, tight junctions

-September 10
10:00 Cell Matrix Interactions – basal lamina
11:00 Epithelial-mesenchymal transition

-September 11
10:00 Angiogenesis
11:00 Innervation

-September 15
10:00 Basal layer stem cells, symmetric versus asymmetric divisions, transient amplifying cells
11:00 Solar radiation, nucleotide excision repair

-September 16
10:00 Basal and squamous cell carcinomas
11:00 Melanoma – biology

-September 17
10:00 Melanoma - treatment
11:00 Contact dermatitis

-September 22
10:00 Other skin disorders
11:00 Other components of skin

-21/7/14 Dr Maria Giuseppina Miano "a functional link between arx and kdm5c genes linked to neuronal diseases defines a crucial epigenetic path"

-16/07/2014 at 14.30 Prof. John F. McDonald "The potential of small regulatory RNAs for the treatment of ovarian cancer

-15/07/2014 ore 14.30-16 Prof.ssa Follenzi “applicazioni terapia genica’’

-30/06/2014 at 14-16 Dott. Cotella ‘’the C-value paradox, junk DNA and ENCODE’’

-27/06/2014 at 14 Manuela Sironi Has nature done the experiment for us? Evolutionary insights into infection susceptibility and autoimmunity

-26/06/2014 at 14 Prof Gianni Del Sal “Disarming mutant P53 in cancer”

-19/06/2014 at 12-13.30 Prof.ssa Follenzi ‘‘terapia genica’’

-12/06/2014 at 14 Gianni Cesareni “Metformin rewires the signaling network of breast cancer cells and changes their sensitivity to growth and apoptotic stimuli”

-11/06/14 at 14 Prof. Fabrizio Loreni "Ribosome alteration in cancer: effect or cause?"

-9/06/2014 at 14 Dott Iacopo Baussano “Assessment of cervical cancer control in Rwanda and Bhutan’’

-5/05/2014 ore 12 Prof. Vittorio Colombo e Dr. Matteo Gherardi, ‘‘atmospheric pressure plasma sources ad processes for biomedical and surface treatment applications’’

-19/03/2014 14.30 Prof Emilio Hirsch ‘‘role of phosphoinositides-3-kinase C2-alph, a Class II PI 3-kinase, in development and cancer’’

-19/02/2014 14 Prof. Salvatore Oliviero Università di Torino ‘‘epigenetic modifications that control stem cell differentiation’’