
Angela Bellini¹, Virginie Bernard¹, Eve Lapouble¹, Nathalie Clement¹, Gaelle Pierron¹, Inge M. Ambros², Katleen de Preter³, Nadine Van Roy³, Ales Vicha⁴, Valérie Combaret⁵, David Betts⁶, Marta Jeison⁷, Smadar Avigad⁷, Martina Morini⁸, Luigi Varesio⁸, Barbara Marques⁹, Annick Muhlethaler¹⁰, Rosa Noguera¹¹, Ana Berbegall¹¹, Jaime Font de Mora¹², Peter F. Ambros², Ruth Ladenstein¹³, Dominique Valteau-Couanet¹⁴, Jean Michon¹, Olivier Delattre¹, Nick Bown¹⁵, Deborah Tweddele¹⁵, Gudrun Schleiermacher¹

¹Institut Curie, Paris, France 
²Children’s Cancer Research Institute, Vienna, Austria 
³Ghent University, Ghent, Belgium 
⁴Charles University, Prague, Czech Republic 
⁵Centre Léon Berard, Lyon, France 
⁶Our Lady's Children's Hospital, Dublin Ireland 
⁷Schneider Children's Hospital, Tel Aviv University, Israel 
⁸Istituto G. Gaslini, Genoa, Italy 
⁹Centro de Genética Humana, Instituto Nacional de Saúde Dr. Ricardo Jorge, Lisboa, Portugal 
¹⁰Institut Universitaire de Pathologie, Lausanne, Switzerland 
¹¹University of Valencia, Valencia, Spain 
¹²Hospital Universitari i Politècnic La Fe, Valencia, Spain 
¹³St Anna Kinderspital, Vienna, Austria 
¹⁴Gustave Roussy, Villejuif, France 
¹⁵Northern Institute for Cancer Research Medical School, Newcastle, UK

Introduction:
In neuroblastoma (NB), activating ALK receptor tyrosine kinase point mutations are detected in 8–10% at diagnosis using conventional sequencing. To determine the potential occurrence and the prognostic impact of ALK mutations in a series of high risk NB patients we studied ALK variation frequencies using targeted deep sequencing in samples of patients enrolled in the SIOOPEN HR-NBL01 study.

Methods:
To date, a series of 326 diagnostic high risk NB samples enrolled in the HRNBL trial has been analyzed, focusing on the exons 23, 24 and 25 containing the F1174, F1245 and R1275 hotspots respectively. DNA was amplified via a two-step PCR approach, the second step consisting of addition of sample-specific barcodes for targeted resequencing in a single experiment. Amplicon sequencing (Illumina HiSeq2500) achieved an extremely high depth over the relevant hotspot (80,000X). The background base variability (error rate) in 32 control samples was 0.017%+/−0.010 at the studied position. Given the mean coverage and error rate, a base frequency >0.06% is significantly different from background noise (Fisher’s exact test).

Results:
At the F1174 hotspot, mutations were observed in 21/326 samples. For 11 cases, these mutations, observed with mutated allele fractions of >20%, occurred at a clonal
level, and in 10 additional cases they occurred at a sub-clonal level (range of mutated allele fractions: 0.207% - 4.195%). At the R1275 hotspot, mutations were observed in 26/326 samples, 12 at a clonal and 14 at a sub-clonal level. All clonal mutations were validated by Sanger sequencing. In one case, 2 sub-clonal ALK mutations (2.997% and 8.943%) were observed at the same position at the hotspot R1275 with another mutation observed at the hotspot F1174. Validation of all mutations observed at a sub-clonal level and analysis of other samples from collaborating groups is currently ongoing. Correlation between these findings and clinical and biological parameters will be established.

Discussion:
Our ongoing study documents a high frequency of clonal (7%) and sub-clonal ALK mutations (7.9%) in high risk NB patients. These findings are of utmost clinical importance given the potential role of ALK mutations in clonal evolution and relapse.