

GENERAL INFORMATION

Project Title

Characterization of a circulating miRNA signature in response to targeted therapy in Diffuse Intrinsic Pontine Glioma (DIPG) patients

Institutions/Units involved in the project

1. Functional Genomics and Bioinformatics Department of Experimental Oncology and Molecular Medicine Fondazione IRCCS Istituto Nazionale dei Tumori - Milano
2. Human Tumors Immunobiology Unit, Department of Experimental Oncology and Molecular Medicine, IRCCS Istituto Nazionale dei Tumori - Milano
3. Pediatric Unit, Fondazione IRCCS Istituto Nazionale Tumori - Milano

Principal Investigator

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Project duration

24 months

Budget

The total budget is 121.000 euro

Abstract (Inglese)

Hypothesis. The present proposal is a translational research to define biomarkers associated with response to therapy and with clinical outcome in diffuse intrinsic pontine glioma (DIPG) patients enrolled in a clinical trial based on radiotherapy, concomitant nimotuzumab and vinorelbine. The main hypothesis of the project is that levels of specific sets of circulating miRNAs are associated with response to treatment and/or with clinical outcome. This hypothesis is supported by robust preliminary data.

Goals. 1. To identify biomarkers by non-invasive and easily accessible methods to improve personalized DIPG management programs. 2. To achieve independent validation of a miRNA signature. This is an essential step to confirm the accuracy of a model in predicting the outcome in DIPG children treated with nimotuzumab/vinorelbine combination. To this end an independent cohort of patients will be explored. 3. To evaluate miRNA expression in longitudinal time point series. 4. To investigate the functional role of the miRNA found in goal 1) by in-vitro cellular models.

Background. DIPG. DIPG, a disease primarily of early school-aged children, is characterized by rapid onset of symptoms in a previously healthy patient, and by pathognomonic MRI findings. Diffuse tumors are currently associated with a very poor prognosis due to their unresectable nature and devastating neurologic symptoms. Radiation is effective as a palliative intervention in a majority of cases, providing transient symptomatic improvement. Without radiation, median survival is approximately 4 months. Subsequent tumor progression is almost common, with median overall survival between 8 and 11 months.

miRNA. miRNAs are single-stranded, non coding RNA molecules of 19-24 nucleotides in length that are generated by sequential cleavage of primary transcripts (pri-miRNAs) and hairloop precursors (pre-miRNAs). To date, more than 2000 human miRNAs have been found according to miRBase and the number of listed miRNAs is increasing. Many studies have so far pointed out that miRNAs expression is deregulated in several different types of tumors. miRNA expression patterns are unique in individual tumors and different between cancer and normal tissues. This specificity and the remarkable stability in a broad range of specimen types (FFPE, plasma and other bodily fluids) make miRNAs useful biomarkers. Recently, expression analysis of circulating miRNA in peripheral blood was demonstrated to be altered in cancer patients, proving their clinical relevance for diagnosis and prognosis.

Clinical significance. At present none biomarker for predicting DIPG prognosis has been entered into the clinical practice. It is essential to develop better tool able to delineate the biological variants of gliomas. Without this approach, treatment targets may be missed and patients receiving toxic therapies not sufficiently targeted to their glioma subtype. Such a biomarker approach means that patients with the same histological diagnosis, tumor location, and co-morbidities may receive differing therapy based on the molecular characteristics of their tumors.

Design and methods. On the basis of our previous experience and availability of clinical samples from an institutional trial, we will assess the expression of circulating miRNA signature predictive of the treatment and then functional role by genetic manipulation.

Abstract (Italiano)

Ipotesi di lavoro. La presente proposta è un progetto di ricerca traslazionale condotto su campioni serici da pazienti con glioma pino intrinseco diffuso (DIPG) arruolata in un trial clinico basato sul trattamento con radioterapia, nimotuzumab e vinorelbina e che ha per obiettivo la definizione di biomarcatori associati all'esito clinico del trattamento. L'ipotesi principale del progetto è che i livelli di espressione di specifici miRNA circolanti siano associati alla risposta al trattamento. Questa ipotesi è supportata da robusti dati preliminari.

Scopo della ricerca. Il progetto si prefigge i seguenti scopi: 1) identificare biomarcatori accessibili con metodi non-invasivi; 2) effettuare una validazione indipendente della firma molecolare identificata; 3) valutare l'espressione dei miRNA circolanti in una serie longitudinale di campioni raccolti durante il follow-up dei pazienti; 4) valutare il ruolo funzionale dei miRNA identificati sfruttando modelli cellulari in-vitro.

Background. DIPG è una patologia che colpisce principalmente bambini/ragazzi in età scolare ed è caratterizzata dal rapido emergere dei sintomi legati alla malattia in soggetti precedentemente sani. Il tumore ha una prognosi estremamente sfavorevole anche in conseguenza dell'impossibilità di intervenire chirurgicamente. La diagnosi avviene attraverso MRI e il trattamento attraverso radioterapia è attualmente la cura primaria ma questo trattamento risulta nella maggior parte dei casi in miglioramenti solo transitori. Senza radioterapia la mediana di sopravvivenza è circa di 4 mesi mentre con radioterapia la mediana di sopravvivenza si attesta tra 8 e 11 mesi. I miRNA sono molecole non codificanti di RNA a singolo filamento di 19-24 nucleotidi generate dal processamento di trascritti primari (pri-miRNAs) e precursori a forma di forcina (pre-miRNAs). Attualmente sono noti più di 2000 miRNA umani. È ormai noto che l'espressione dei miRNA viene deregolata nei tumori e la possibilità di rilevarli in diversi tipi di campioni incluse le biopsie liquide (plasma, siero, urine, saliva) rende queste molecole estremamente interessanti ai fini dell'identificazione di marcatori biomolecolari. Recentemente l'analisi dell'espressione dei miRNA circolanti presenti nel sangue si è dimostrato alterato in pazienti affetti da diverse patologie oncologiche.

Implicazioni nella clinica. Attualmente non esistono biomarcatori capaci di predire la prognosi in pazienti DIPG. È essenziale sviluppare migliori strumenti diagnostici/prognostici in quanto la loro assenza può portare al trattamento inutile di soggetti che invece potrebbero beneficiare di trattamenti alternativi. Pertanto questo approccio comporta che tumori con la stessa diagnosi, localizzazione e comorbidità possano essere indirizzati verso trattamenti specifici diversi basati sulle caratteristiche molecolari del tumore.

Disegno dello studio e metodi. Sulla base della esperienza come messo in evidenza nei risultati preliminari e della disponibilità di campioni da un trial, valuteremo l'espressione dei miRNA circolanti e il loro ruolo funzionale.

Background and rationale

Diffuse intrinsic pontine glioma (DIPG), a disease primarily of early school-aged children, is characterized by rapid onset of symptoms in a previously healthy patient, and by pathognomonic MRI findings. Without radiation, median survival is approximately 4 months (Lassman , 1967). Radiation is effective as a palliative intervention in a majority of cases, providing transient symptomatic improvement. Subsequent tumor progression is almost universal, with median overall survival between 8 and 11 months, and overall survival of approximately 30% at 1 year and less than 10% at 2 years (Hargrave , 2006; Bradley, 2013). Rare (2–3%) long-term survival has been reported, usually associated with atypical imaging and clinical features at presentation (Pollack et al, 2011; Jackson et al, 2013). Diffuse tumors constitute the majority (75–80%) of brain tumors and are associated with a very poor prognosis due to their unresectable nature, devastating neurologic symptoms associated with the local extension, and poor response to adjuvant therapy. The median time to tumor progression is 5–6 months, with a median survival of 9–12 months despite treatment (Freeman et al, 1998).

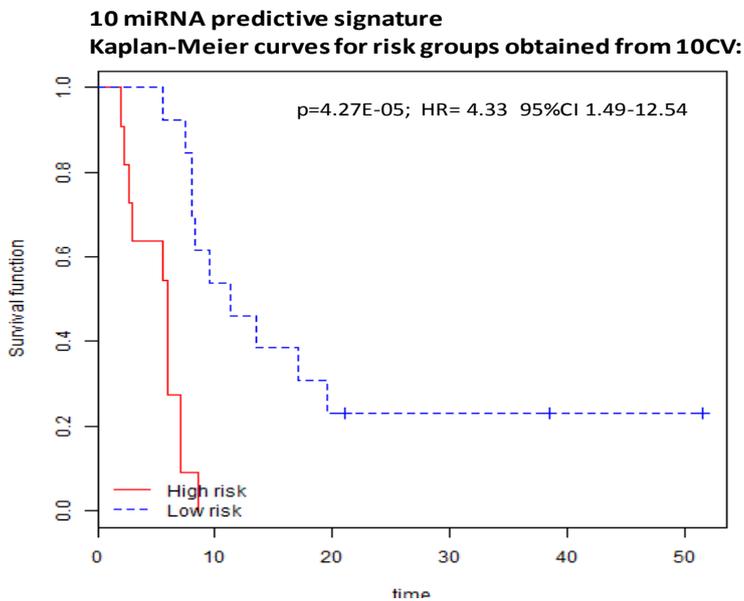
At present none biomarker for predicting prognosis has been entered into the clinical practice. It is essential to develop better tools able to delineate the biological variants of gliomas. Without this approach, treatment targets may be missed and patients given toxic therapies not sufficiently targeted to their glioma subtype. Such a biomarker approach means that patients with the same histological diagnosis, tumor location, and co-morbidities may receive differing therapy based on the molecular characteristics of their tumors.

miRNAs are single-stranded, non coding RNA molecules of 19-24 nucleotides in length that are generated by sequential cleavage of primary transcripts (pri-miRNAs) and hairloop precursors (pre-miRNAs)(Kusenda, 2006). To date, more than 2000 human miRNAs have been found according to miRBase and the number of listed miRNAs is increasing (miRBase: www.miBase.org). Many studies have so far pointed out that miRNAs expression is deregulated in several different types of tumors (Bardel, 2011). miRNA expression patterns are unique in individuals tumors and different between cancer and normal tissues (Volinia, 2006). This specificity and the remarkable stability in a broad range of specimen types (FFPE, plasma and other body fluids) make miRNAs useful biomarkers (Cortez, 2011). Recently expression analysis of circulating miRNA in peripheral blood was demonstrated to be altered in cancer patients, proving their clinical relevance for diagnosis and prognosis (Zhu, 2009). The relationship between circulating miRNAs and cancer tissue was first investigated by Mitchell et al (Mitchell, 2008) showing that circulating miRNAs originate from cancer tissues. This study, supported by others, demonstrate that the level of circulating miRNAs reflects the miRNome of tumor tissue, providing evidence on the hypothesis that circulating miRNAs can serve as ideal biomarkers. Further studies elucidated how circulating miRNAs are actively secreted into the blood stream. Increasing proofs support the idea that circulating miRNA are incorporated into exosomes or are bound to RNA-binding proteins, explaining their stability against RNA digestion (Valadi, 2007).

Currently a variety of miRNA detection methods including northern blotting, in situ hybridization, qRT-PCR, microarrays and deep sequencing are available. The procedures for detection of circulating miRNAs are the same as that for cellular miRNAs, except for the extremely low amount of starting material. High-throughput profiling techniques such as microarray and miRNA deep sequencing are effective tools to obtain expression profiles. Since these methods generally required a relative large volume of blood, they are used for the initial discovery screening. Then for validation approaches, qRT-PCR methodologies have proved to be useful in detecting the low level of circulating miRNA expression.

Preliminary results

With the aim of exploring add-on strategies, a non-randomized, open label phase II pilot study was conducted at Fondazione IRCCS Istituto Nazionale dei Tumori (Milan) to assess the efficacy in terms of objective response rate according to the RECIST criteria of combining nimotuzumab and vinorelbine with radiation in newly-diagnosed DIPG. Vinorelbine was administered at 20 mg/m² weekly together with nimotuzumab at 150 mg/m² during the first 12 weeks of treatment, when



radiotherapy was delivered from week 3 to 9 at total dose of 54 Gy. Vinorelbine at 25 mg/m² was given any other week, with the same dose of nimotuzumab, thereafter until tumor progression or for a total of two years. Twenty-five children (median age 7.4) were enrolled according to the standard MRI inclusion criteria and a follow-up with a median observation time of 21 months was achieved. A response was observed in 24/25 patients (96 %). One-year PFS rates was 30 ± 10 % respectively; the median PFS were 8.5 months.

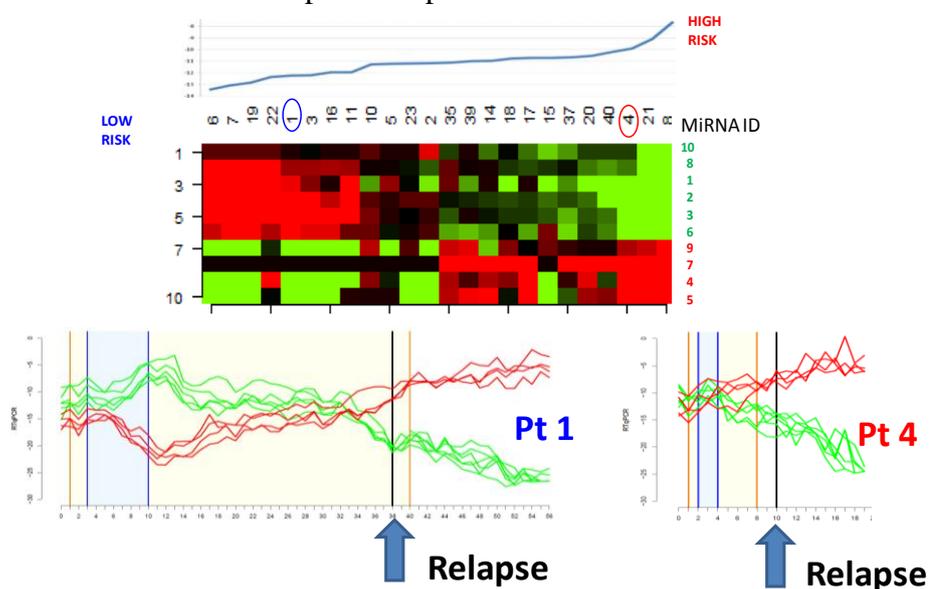
Serum specimens were collected at baseline and during follow-up. We

used a high-throughput microRNA screening approach to assess miRNA profile in serum samples obtained from 24 DIPG patients with the main aim to identify novel non-invasive biomarkers able to improve the prediction of disease outcome and therapeutic sensitivity. Here we present the preliminary results of serum miRNA profiling at baseline. microRNA expression profiling was performed using Agilent platform and Human miRNA SureSelect 8x60K containing 2006 miRNAs annotated on miRBase19.0. Primary data analysis yielded a matrix containing 330 detectable miRNA. Association with PFS allowed us to disclose a signature of 10 miRNAs able to stratify high and low risk patients (HR=4.33, 95%CI 1.49-12.54; $p=4.27E-05$).

The 10-miRNA signature was validated by RTqPCR and its accuracy in predicting the response to treatment in DIPG patients was confirmed. At present, this is the first study that aims at investigating circulating miRNA levels in DIPG pediatric patients.

Two patients were selected for evaluation of the 10 miRNA expression in longitudinal analysis (up to 30 serum samples for each patient, collected from diagnosis till relapse or last control) and associated to clinical data.

Primers were obtained from Exiqon (Vedbæk, Denmark). qRT-PCR was performed using



the miRCURY LNA™ Universal RT microRNA PCR system (Exiqon) and following the manufacturer's instructions. Real-time was run using QuantStudio 12flex. Normalization was performed using has-miR16 and 3 invariant miRNAs identified in the microarray profiling. Results are reported as $-\Delta Ct$. Shaded orange area identifies the antibody/vinorelbine treatment. Shaded blue area identifies the radio therapy Black bar identifies relapse. The preliminary results were promising since different patterns of miRNA expression were recorded between low and high risk patients suggesting that the relative expression of the signature could potentially predict disease outcome.

Study aims.

In order to evaluate the accuracy of the miRNA signature to foresee treatment outcome in DIPG patients, the following activities are required:

- A) independent validation of our miRNA signature. This is an essential step to confirm the accuracy of our model in predicting the outcome in DIPG children treated with nimotuzumab/vinorelbine combination. An independent cohort of patients is required to this aim. We will use the serum specimens collected in the frame of "*phase II open label randomized study of radiotherapy, concomitant nimotuzumab and vinorelbine and re-irradiation at relapse versus radiotherapy and multiple elective radiotherapy courses with concomitant nimotuzumab and vinorelbine for newly diagnosed childhood and adolescence DIPG*" (PI Dr. M. Massimino).
- B) Evaluation of predictive power of our miRNA signature. Our signature will be challenged against a cohort of children not affected by neurological pathologies or affected by neurological pathologies but not treated with nimotuzumab and vinorelbine match by age and gender. This is an essential point to ascertain the accuracy of our signature to predict the response to nimotuzumab and vinorelbine treatment in DIPG patients. In alternative we will assess the accuracy of our miRNA model to be a prognostic tool of neurological pathologies in children.
- C) Evaluation of miRNA expression in longitudinal time point series. First of all, we will extend the number of cases under evaluation. Based on the expression during time we will monitor the efficacy of treatment and the ability to predict the time of relapse.
- D) Functional role of miRNA. Most of the miRNA present in our signature were only recently annotated in miRBase (miRBase_v19.0; August 2013). Really few information is available on their biological functions. For this reason we propose to investigate their functional role exploiting *in vitro* functional models.

Expected Results

The promising utility of liquid biopsies has recently gained great attention toward its clinical application in the management of cancer patients. As matter of fact, to improve the clinical outcome of DIPG patients, accurate detection and monitoring of disease are necessary. At present, surgical and/or biopsy specimens are generally used to detect tumor alterations and to build treatment predictive models. However, the collection of this kind of material due to its invasive nature is not feasible in DIPG children. In addition, the tumor at baseline may fail to reflect the current tumor dynamics and drug sensitivity that may change during the therapeutic process. Therefore, liquid biopsies monitoring the presence of cell free miRNAs could be the ideal material for developing non-invasive biomarkers helping to define personalized DIPG management programs.

Personnel involved into the project

NAME	Time for the project (%)	Role on project
Loris De Cecco (03/04/73) PI	50	Biologist, PhD, he has wide experience in high-throughput methodologies. He will take care of project coordination. He will oversee the experimental activities and will contribute to the computational analyses, predictor design/refinement. He will coordinate the UNIT-A of the project.
Silvana Canevari (30/11/1948) Internal collaborator	30	Biologist, responsible of Functional Genomics core facility, will oversee and coordinate the activities and the genomic aspects. She will participate on the activities of UNIT-A.
Maura Massimino (8/03/1962) Internal collaborator	30	MD, Oncologist, director of the pediatric unit. She will contribute to the planning project. She is responsible of the “phase II open label randomized study of radiotherapy, concomitant nimotuzumab and vinorelbine and re-irradiation at relapse versus radiotherapy and multiple elective radiotherapy courses with concomitant nimotuzumab and vinorelbine for newly diagnosed childhood and adolescence DIPG”. She will coordinate the UNIT-B of the project.
Andrea Anichini (19/02/1952) Internal collaborator	30	Biologist, head Human Tumors Immunobiology Unit, he will oversee the functional genomics analyses. He will coordinate the UNIT-C of the project.
Marco Giannoccaro (01/09/1986) Fellow	100	He has experience in molecular biology and in high-throughput methodologies and he will perform the experimental activities needed in the project. He will participate on the activities of UNIT-A.
TBD () Fellow	100	Experience in molecular biology and cellular biology methodologies. He/she will perform the functional activities needed for the project. He/She will participate on the activities of UNIT-B.

The principal investigator and all other personnel of the research team have already successfully collaborated. Evidence lines in the recent paper on nimotuzumab and vinorelbine treatment of DIPG

patients (Massimino et al. Results of nimotuzumab and vinorelbine, radiation and re-irradiation for diffuse pontine glioma in childhood. J Neurooncol. 2014 Jun;118:305-12). In addition the results were presented at the EACR-AACR-SIC conference (Florence , 20-23 June 2015).

BRIEF CURRICULUM: Maura Massimino, MD

Maura Massimino was born on March 8, 1962.

She graduated in Milan University in 1987 with laude. She achieved Hematology Board in 1991 and Pediatrics Board in 1995 at Milan University. From 1987 onwards she worked at the Pediatric Oncology Unit of Istituto Nazionale Tumori of Milan, first as resident until 1989, afterwards as permanent staff member, from May 2009 as Deputy Director and from July 2010 as Director. Her clinical research is particularly devoted to childhood brain tumors, lymphomas and thyroid cancer. Co-chair for the SIOP brain tumor subcommittee of the Ependymoma study group since 2007, chair since 2012. She is also involved in late effects evaluation both from the endocrinological and neuro-cognitive point of views. She is the author of more than 200 published full papers, more than 350 meeting communications and several book chapters. She is married, mother of two children.

BRIEF CURRICULUM: Silvana Canevari, PhD

Born on November 30, 1948

Graduated in Milan University in 1972 with laude, PhD in Cancer Immunology in 1974. From 1973 onwards she worked at the Experimental Department of Fondazione IRCCS Istituto Nazionale Tumori of Milan, first as fellow until 1974, afterwards as permanent staff member. From 2008 to 2013 she was Director of the Molecular Therapies Unit and afterward she works as associate researcher to Scientific Direction for the facility of Functional Genomics and Bioinformatics. Her experimental research was devoted initially to the immunological characterization and subsequently to the molecular characterization of human tumors. Since 2007 she is responsible of The Functional Genomics and Bioinformatics facility. She is the author of more than 200 published full papers, more than 350 meeting communications and several book chapters concerning different aspects of the experimental oncology, immunology, biochemistry and biotechnology applied to human tumors.

BRIEF CURRICULUM: Andrea Anichini, PhD

Born on November February, 2nd , 1952

Dr. Anichini is member of the Italian Society of Cancerology and of EACR. He has published 147 papers on peer reviewed journals and has an H index=37. He acts as reviewer for international science journals, including Science Transl. Med., J. Exp. Med., Cancer Res. and other journals. He has been awarded grants from AIRC and from Italian and European funding agencies since 1992. He is mentoring four fellows. He has devoted most of the past three decades to research in tumor immunobiology with emphasis on melanoma and B cell lymphomas. He has contributed to identification of anti-tumor HLA-restricted T cells in melanoma patients, identification of melanocyte lineage antigens as immunogenic epitopes, understanding of development of antitumor immunity during progression, molecular identification of tumor antigens and mechanism of

response to anti-CTLA-4. Dr. Andrea Anichini is author of 147 peer-reviewed publications and 24 book chapters concerning different aspects of the biology and immune response to human tumors.

BRIEF CURRICULUM: Marco Giannoccaro

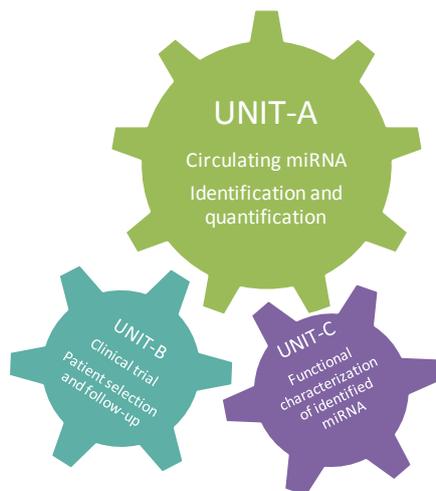
Born on November September, 1st , 1986

Dr. Giannoccaro, as member of Functional Genomics Unit at the Fondazione IRCCS National Cancer Institute, is responsible for the extraction of nucleic acids from frozen tissue, FFPE and biofluids. In addition he is responsible of quality control through the use of RTqPCR QuantStudio12K-flex platform in order to ensure adequate quality for microarray analysis. He has good experience in processing samples for microarray analysis (labeling, purification), miRNA and gene-expression profiling by Agilent and Illumina platforms and gene-expression DASLwg technology for FFPE samples.

BRIEF CURRICULUM: TBD

Degree in biology or biotechnology. Young researcher with experience in microarray, high-throughput methodologies, including data analysis, molecular biology, and cellular biology methodologies.

PERT chart (Program Evaluation Review Technique)



METHODS

The present proposal is a translational research linked to “phase II open label randomized study of radiotherapy, concomitant nimotuzumab and vinorelbine and re-irradiation at relapse versus radiotherapy and multiple elective radiotherapy courses with concomitant nimotuzumab and vinorelbine for newly diagnosed childhood and adolescence DIPG”. Collection of blood specimens will be carried out at baseline (before treatment) and during follow-up.

Here below the main features of the trial are summarized

Inclusion criteria

1. Patients from 2 to 21 years old will be eligible;
2. No previous treatment consented apart from steroids;
3. Strict eligibility criteria will radiologically-verified DIPG (an intrinsic, pontine-based infiltrative lesion hypointense on T1- and hyperintense on T2-weighted sequences, involving at least 2/3 of the pons) (Hargrave et al, 2006);
4. symptoms lasting less than 6 months, life expectancy \geq 4 weeks; Karnowski/Lansky performance status \geq 40%;
5. no organ dysfunction; no pregnancy or breast-feeding;
6. Patients undergo baseline cranial MRI with gadolinium, to be repeated if treatment begins more than 2 weeks; spinal MRI due to the occurrence of metastatic cases at diagnosis will also be mandatory;
7. Written and signed informed consent from parents or legal guardians will be obtained before starting the treatment.

Treatment design

Pre-therapy examinations (within 14 days prior to the start of therapy):

- Cranial and holospinal MRI with gadolinium (estimation of index lesion);
- Full clinical examination including neurological, body weight, height, Karnowski or Lansky performance status;
- Full blood count, creatinine, electrolytes, Ca, Mg, phosphate, AST, LDH, total protein, albumin, CRP, bilirubin, blood sugar; coagulation tests (Quick value or INR, PTT, TT);
- Pregnancy test in females of childbearing age (must be negative).
- Evaluation of quality of life and life situation.

Standard arm: medical treatment

Nimotuzumab + Vinorelbine and full-dose radiotherapy: standard arm

Nimotuzumab 150 mg /m²/d as iv short-term infusion for 30 min weekly in week 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12 and Vinorelbine 20 mg/m²/d weekly in week 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12 as iv short-term infusion for 30 min (Induction phase);

1st re-evaluation week 12 (day 84-92). In case of non-progressive disease: Nimotuzumab 150 mg/m²/d iv short-term infusion for 30 min and Vinorelbine 25 mg/m²/d as iv short-term infusion for 30 min biweekly in week 14, 16, 18, 20, 22, 24 (Consolidation phase I);

2nd re-evaluation week 24, thereafter in case of non-progressive disease: Nimotuzumab 150 mg/m²/d iv short-term infusion for 30 min and Vinorelbine 25 mg/m²/d as iv short-term infusion for 30 min biweekly, with reevaluation at week 36 until progression or maximum at week 104

Experimental arm

Pre-therapy examinations like in standard arm.

Nimotuzumab + Vinorelbine and refracted radiotherapy doses: experimental arm

Nimotuzumab 150 mg/m²/d as iv short-term infusion for 30 min weekly in week 1, 2, 3, 4, 5, 6,7, 8,9,10, 11, 12 and Vinorelbine weekly 20 mg/m²/d in week 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12 as iv short-term infusion for 30 min (Induction phase, as for standard arm);

1st re-evaluation week 12. In case of non-progressive disease, any other week, Nimotuzumab 150 mg/m² as iv short-term infusion for 30 min and Vinorelbine 25 mg/m²/d as iv short-term infusion for 30 min for a total duration of 104 weeks;

2nd re-evaluation week 24, thereafter in case of non-progressive disease re-irradiation one for a total of 19.8 Gy in 11 fractions at 1.8 Gy/day from week 25 to week 27 together with vinorelbine/nimotuzumab continuation any other week;

3rd re-evaluation week 36, thereafter in case of non-progressive disease vinorelbine/nimotuzumab continuation any other week;

4th re-evaluation week 44, thereafter in case of non-progressive disease: re-irradiation two for a total of 19.8 Gy in 11 fractions at 1.8 Gy/day from week 45 to week 47 together with vinorelbine/nimotuzumab continuation any other week;

Further re-evaluation will be done at week 60 and thereafter any 12 weeks as for standard arm continuing vinorelbine and nimotuzumab until progression or until full duration of treatment for 104 weeks. Patients will continue with re-irradiation courses also in case of progressive disease, and will continue to be evaluated for OS. The intended sample size is N = 54 patients. A stratified randomization procedure will be used to randomize eligible, consenting patients to experimental or control arms (1:1 ratio). Randomization will occur using a computer generated randomized number sequence. Stratification factors, demonstrated to be significantly associated with patient outcome in a previous study (Massimino 2014) will be patients age (≤ 4 years age, >4 years) and need for shunting at diagnosis (no, yes).

Collection of serum specimens.

Serum provides the liquid portion of the blood without cells and clotting factors and, therefore, should contain proteins and other molecules that represent the whole body system. The cells and clotting factors must be removed from the blood sample by allowing adequate time for a clot to form. Most manufacturers of collections systems for serum samples recommend 30–60 min at room temperature for a clot to form and longer if the subject was taking any kind of anticoagulant at sample collection

Blood from initial diagnosis, from specific time points during treatment, and during follow-up, will be collected. The blood sample are/will be processed within 1 h after collection by centrifugation at 1,300 x g at 4°C for 20 min, and plasma separated in aliquots in fresh tubes and stored at -80°C.

The samples will be sent to the Functional and Bioinformatics (INT) for RNA extraction and profiling.

miRNA profiling

The miRNA microarray experiments will be performed by the Functional Genomics of the Department of Experimental Oncology and Molecular Medicine (Fondazione IRCCS Istituto Tumori) using Human miRNA SurePrint Microarray (Agilent Technology). Since the number of miRNAs is still increasing with the everyday discovery of new miRNAs, microarrays are at present one of the most used methodologies for assessing the expression of miRNAs in tissue samples, enabling the cheap and reliable profiling on the last available miRBase version. For each miRNA, multiple probes are spotted on the array. The average intensity of these probes will be calculated and used as expression value of the miRNA for further analyses. For each plasma sample, total RNA will be extracted, labeled, hybridized with the miRNA array and further processed following the manufacturer's protocols. The arrays will be scanned using an Agilent Technology G2565CA scanner and the scanned images will be processed using the Feature Extraction software package version 10.5 (Agilent Technology) in order to extract the raw data. Validation in an independent cohort of samples of our 10 miRNA signature will be carried out. Moreover we will test the miRNA expression in autopsy material that has undergone only a small amount of postmortem degradation.

Data analysis

Analysis will be performed using BrB-Array Tools v4.2.1 stable release developed by Dr. Richard Simon (NCI) and BRB-Array Tools development team (EMMES corporation). miRNAs expression will also be related to clinical outcome; the analyses will be performed separately for each selected miRNA. Time will be calculated as the interval between patient randomization and disease progression or death, whichever will occur first, with censoring at the date of last follow up control for patients alive and without documented progression. We will use a multivariable Cox proportional hazard model in which miRNA expression will be evaluated as continuous variable; the clinical and histological parameters will be included in the model as adjustment factors. The performance of the model including the miRNA under evaluation in terms of discriminative ability will be evaluated by calculating the bootstrap corrected Harrell C statistic and compared with that of the model not including the miRNA, with the aim of evaluating whether the expression data provides more accuracy than that associated to clinical/histological parameters only.

In vitro models

As neoplastic tissues from these patients are not available, the possibility of carrying out functional studies with DIPG cell lines would strongly improve the quality/meaning of the results that we are obtaining by analysis of serum samples. To this end, we plan to exploit DIPG cell lines to:

- i) assess the miRNA content in exosomes derived from the cell lines.

ii) silence/overexpress miRNA of interest in cell lines for functional characterization. To this end, we will analyze changes in gene expression (by whole genome expression analysis), survival, and proliferation (by apoptosis/proliferation assays) of the cell lines where miRNA of interest have been silenced or overexpressed.

Timetable

UNIT	Aim	Activities	1° year				2° year			
UNIT-A	A) Independent validation	RNA extraction; quality check and microarray analysis of the entire case material	■	■	■	■				
		Bioinformatics analysis of microarray data			■	■	■	■		
		Statistical analysis					■	■		
		Validation of microarray data by qRT-PCR						■	■	■
	B) Evaluation of predictive power of our signature	Collection of sera specimens	■	■	■	■				
		Microarray analysis of validation set		■	■	■				
		Bioinformatics analysis of microarray data				■	■	■		
		Statistical analysis					■	■	■	
	C) Evaluation of miRNA expression in longitudinal series	Integration of genomic and clinical data						■	■	■
		RNA extraction	■	■	■	■				
		qRT-PCR analysis of our 10 miRNA signature			■	■	■			
		Bioinformatics analysis of microarray data			■	■	■			
UNIT-B	D) Clinical trial	Statistical analysis to develop model associated to type and time of treatment					■	■	■	■
		selection of patients	■	■	■	■				
		treatment	■	■	■	■				
		longitudinal blood withdrawal	■	■	■	■	■	■	■	■
UNIT-C	D) Functional role of selected miRNAs	patients' follow-up	■	■	■	■				
		exosome extraction form blood and cell cultures	■	■	■	■				
		genetic manipulation of cell cultures: knock-in			■	■	■	■	■	■
		genetic manipulation of cell cultures: knock-out			■	■	■	■	■	■

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Budget

	1st year	2nd year	Total
Personell costs	38,000	38,000	76,000
Consumables	15,000	15,000	30,000
Meeting and travel costs	1,000	1,000	2,000
Other (publication costs)	1,000	1,000	2,000
subtotal total cost/year	55,000	55,000	
		subtotal	110,000
Overheads (adminstration office)10%	5,500	5,500	
		Total cost	121,000

i. **Personnel costs:** Two fellowships; 38.000,00 Euro/year (**tot 76.000,00 euro**)

ii. **Supplies/consumables:** miRNA microarray chips and kits: 5.000 euro/year; miRNA extraction kits: 2,000 euro/year; RTqPCR assays and reagents: 4,000 euro/year; reagents for cell culture: 4,000 euro/year (**tot. 30.000,00 euro**)

iii. **Equipment:** none to be imputed to this grant

iv. **Patient care costs:** none

- 2004 – 2007 PhD programme (Open University) under supervision of Dr. M. Pierotti. Istituto Nazionale Tumori, Milan
- 1999 – 2003 FIRC and AIRC fellowship, Molecular genetics of cancer unit, IFOM (Fondazione FIRC di Oncologia Molecolare), Milan. Director: Dr M.Pierotti

Since 2000 Dr. De Cecco's scientific activity has been focused on the analysis of gene expression patterns underlying the progression and the clinical outcome in different types of tumors. He has an extensive experience and background in molecular biology techniques and microarray procedures, including microarray manufacturing, synthesis of fluorescent probes, and hybridization. Since 2007 as member of the Functional Genomics Unit at Fondazione IRCCS Istituto Nazionale Tumori, he has carried out microarray studies using the Illumina and Agilent platforms and has a deep knowledge in protocols for gene-expression, for miRNA profiling and methylation analysis. In addition he has a long experience in handling and processing microarray data, including excellent skills in the use of microarray tools for data analysis (BrB-Arraytools, MeV, R Bioconductor) and for pathway identification (DAVID, GeneSpring GX, Partek Genomic Suite, Ingenuity Pathway Analysis, CytoScape). He has experience in whole-exome NGS analysis with SOLiD (LifeTechnologies) platform, including library construction and sequencing. He is co-author of more than 40 scientific papers.

Awards: gold medal as young scientist for his contribution on cancer research by gene-expression analysis using high-throughput cDNA microarray platforms (2006; Associazione Amici di Milano).

Grants: "Rule of IL2-family members in CLL" founded by Compagnia di S.Paolo Foundation (Genoa) (2012; 10.000 euro).

Role in the project: Dr. De Cecco will oversee and coordinate all the activities, he will be responsible for study design and for the planning and development of the research; he will oversee the bioinformatics analysis required for the project and he will maintain the relationships with all collaborators. As PI, he will be full dedicated to the project and he will be last author of the publications related to the results of the project.

Papers indexed in PubMed (2010-2015):

In the last 5 years, the PI is first author of 8 papers as first author and 1 paper as last author. **Total I.F. =158**

1: Triulzi T*, **De Cecco L***, Sandri M, Prat A, Giussani M, Paolini B, Carcangiu ML, Canevari S, Bottini A, Balsari A, Menard S, Generali D, Campiglio M, Di Cosimo S, Tagliabue E. Whole-transcriptome analysis links trastuzumab sensitivity of breast tumors to both HER2 dependence and immune cell infiltration. *Oncotarget*. 2015 Jul 1.

2: **De Cecco L**, Capaia M, Zupo S, Cutrona G, Matis S, Brizzolara A, Orengo AM, Croce M, Marchesi E, Ferrarini M, Canevari S, Ferrini S. Interleukin 21 Controls mRNA and MicroRNA

Expression in CD40-Activated Chronic Lymphocytic Leukemia Cells. *PLoS One*. 2015 Aug 25;10(8):e0134706.

3: Vitali C, Bassani C, Chiodoni C, Fellini E, Guarnotta C, Miotti S, Sangaletti S, Fuligni F, **De Cecco L**, Piccaluga PP, Colombo MP, Tripodo C. SOCS2 controls proliferation and stemness of hematopoietic cells under stress conditions and its deregulation marks unfavourable acute leukemias. *Cancer Res*. 2015 Apr 9.

4: **De Cecco L**, Nicolau M, Giannoccaro M, Daidone MG, Bossi P, Locati L, Licitra L, Canevari S. Head and neck cancer subtypes with biological and clinical relevance: Meta-analysis of gene-expression data. *Oncotarget*. 2015 Mar 20.

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5: **De Cecco L***, Negri T*, Brich S, Mauro V, Bozzi F, Dagrada G, Disciglio V, Sanfilippo R, Gronchi A, D'Incalci M, Casali PG, Canevari S, Pierotti MA, Pilotti S. Identification of a gene expression driven progression pathway in myxoid liposarcoma. *Oncotarget*. 2014 Aug 15;5(15):5965-77.

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7: Massimino M, Biassoni V, Miceli R, Schiavello E, Warmuth-Metz M, Modena P, Casanova M, Pecori E, Giangaspero F, Antonelli M, Buttarelli FR, Potepan P, Pollo B, Nunziata R, Spreafico F, Podda M, Anichini A, Clerici CA, Sardi I, **De Cecco L**, Bode U, Bach F, Gandola L. Results of nimotuzumab and vinorelbine, radiation and re-irradiation for diffuse pontine glioma in childhood. *J Neurooncol*. 2014 Jun;118(2):305-12. doi: 10.1007/s11060-014-1428-z. Epub 2014 Apr 3.

8: Minna E, Romeo P, **De Cecco L**, Dugo M, Cassinelli G, Pilotti S, Degl'Innocenti D, Lanzi C, Casalini P, Pierotti MA, Greco A, Borrello MG. miR-199a-3p displays tumor suppressor functions in papillary thyroid carcinoma. *Oncotarget*. 2014 May 15;5(9):2513-28.

9: Dassano A, Colombo F, Trincucci G, Frullanti E, Galvan A, Pettinicchio A, **De Cecco L**, Borrego A, Martinez Ibañez OC, Dragani TA, Manenti G. Mouse pulmonary adenoma susceptibility 1 locus is an expression QTL modulating Kras-4A. *PLoS Genet*. 2014 Apr 17;10(4):e1004307.

10: Granata A, Nicoletti R, Tinaglia V, **De Cecco L**, Pisanu ME, Ricci A, Podo F, Canevari S, Iorio E, Bagnoli M, Mezzanzanica D. Choline kinase-alpha by regulating cell aggressiveness and drug sensitivity is a potential druggable target for ovarian cancer. *Br J Cancer*. 2014 Jan 21;110(2):330-40.

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Milan, 10 September 2015

TO WHOM IT MAY CONCERN

I confirm that the “phase II open label randomized study of radiotherapy, concomitant nimotuzumab and vinorelbine and re-irradiation at relapse versus radiotherapy and multiple elective radiotherapy courses with concomitant nimotuzumab and vinorelbine for newly diagnosed childhood and adolescence DIPG” is an academic study approved by the Institutional Ethical Committee and Scientific Directorate. The costs relative to insurance, imaging and centralized radiological revisions of the cases are covered by charitable contributions.

Sincerely,

Maura Massimino, MD.
Director of Pediatric Oncology Unit
Fondazione IRCCS Istituto Nazionale dei Tumori
Via Venezian 1,
20133 Milan, Italy