Section 1

AISS Applicant: Roberto Valli

Application page 1

Associazione Italiana Sindrome di Shwachman-Diamond (AISS)
Via Pioveghetto 15, - 35136 Padova
Tel - FAX +39 049 8736130
E-mail: aiss@shwachman.it

Shwachman-Diamond Syndrome Italian Research Grant

Maximum Amount euro 10,000,00

Firm Deadline for Receipt of Applications: 3rd March 2018

Eligibility: Persons applying for these grants if not in a faculty position need to provide a declaration by a supervisor with a position in the department (not a training position) and with authority to hold an independent research grant.

Terms of Support: Support may be provided for one (1) year in an amount not to exceed E 10,000. Indirect costs are permitted and are not to exceed 10% of the total costs. The AISS will provide preference to those applications in which funds are used for supplies, equipment, technicians and other expenses and not for support of the salary of the PI or co-PIs.

Review: All applications will be reviewed by the AISS Scientific Committee (AISS-SC) or its designees.

Application: The application contains two sections.

Section 1, forms attached. The applicant and co-applicants must also include a current curriculum vitae. Section 2: Research Plan, divided as indicated below. Parts A through D should not exceed 6 pages, using a font no smaller than 10 point.

Part A. Specific aims
Part B. Significance and background
Part C. Preliminary studies
Part D. Experimental design and methods
Part E. References (not included in the 6 page limit)
Part F. Relevance of the research to Shwachman-Diamond Syndrome
Part G. For junior faculty separate letter from supervisor or department head confirming commitment to project, and to provision of space and facilities
Part K. If human subjects and animals are involved, a statement by the PI or supervisor overseeing human or animal studies is compulsory. If considered as necessary by the AISS-SC, more information about ethical committee study approval may be asked.

Submission by email to the AISS: aiss@shwachman.it
1. Title of Proposal:

Deletions of the long arm of chromosome 20 in bone marrow: aCGH, expression analysis and development of a cellular model by the use of HL-60 cell line by the CRISPR/CAS9 system.

2. Applicant Information:

Name: Roberto VALLI

Title and Degree(s):
Researcher in Medical Genetics, University of Insubria. Biological sciences degree. PhD in Congenital and Acquired Degenerative Diseases

Work Address:
Dipartimento di Medicina e Chirurgia
Università dell'Insubria
Via J.H. Dunant, 5
21100 VARESE

Telephone: +39 0332 217112

FAX: +390 332 217119

Email: roberto.valli@uninsubria.it

3. Applicant Curriculum Vitae: beginning on the next page, with 2 page limit. This will form application pages 3 and 4.
Curriculum Vitae

- Born in Varese (Italy) on February 8th, 1973
- In 2001 he graduates in Biological Sciences with a score of 110/110 summa cum laude disserting an experimental thesis work titled "Cytogenetic characterization of Chromosome 10 breakpoint's in the t(10;21)(q23.1;q11.2) translocation associated with a patient affected by a rare form of congenital heart disease"
- In December 2001 he starts the PhD course in "Congenital and Acquired Degenerative Diseases" of the University of Insubria, working in the Medical Genetic lab of the Clinical and Experimental Biomedical Sciences Department in Varese.
- Since 2002 is member of the Italian's Human Genetics Society (S.I.G.U.)
- During the PhD studies, he works for a period in the Biology and Genetics labs of the D.A.P.E.G. Department of the University of Bari (Italy)
- In 2005 he doctorates disserting the experimental works titled "Molecular and Citogenetical Characterization of familiar t(12;15) translocation".
- In November 2005 he starts the Post-doc on the "Mechanism of origin and molecular definition of structural chromosomal anomalies" working in the Medical Genetic lab of the Clinical and Experimental Biomedical Sciences Department in Varese.
- In December 2005 he achieved the qualification to practise the Biologist profession, passing the related Italian examination.
- Since 2001 he carries out integrative didactics, by substituting the official professors in the courses of "Genetics and Biology" and "Medical Genetics" for the degree courses of the Faculty of Medicines and Surgery of the University of Insubria.
- In October 2008 he becomes a researcher in Medical Genetics for the Medical and Surgery Faculty of the University of Insubria and works in the Medical Genetic lab of the Clinical and Experimental Biomedical Sciences Department in Varese.
- Since 2009 he is teacher in general biology or medical genetics in 6 degree courses of the Insubria’s School of Medicine (formerly Faculty of Medicine and Surgery)
- Since 2014 is member of the European Cytogeneticists Association

Research Interests

The principal research lines in field of Medical Genetics were and still are:
- Studies on karyotype/phenotype correlation in constitutional and acquired chromosome anomalies through the use of cytogenetic, molecular cytogenetics, array comparative genomic hybridization (aCGH) and molecular genetics tools.
- Studies on the chromosome variability in leukaemia and, in particular, in myelodysplasia and myeloproliferative disorders at the diagnosis, during the course of disease, and after bone marrow transplantation.
- Studies on families with Mendelian diseases associated to myeloproliferative disorders
- Studied on Shwachman-Diamond Syndrome, and other inherited bone marrow failure syndromes predisposing to myelodysplastic syndromes and myeloproliferative disorders.
Studies on chromosome abnormalities with gene effects leading to peripheral cytopenias (uni-, bi, and tri-linear) and/or bone marrow aplasia/hypoplasia.

Studies on the sensitivity of the array comparative genomic hybridization (aCGH) and its implications in acquired chromosome imbalances.

Study of the correlation between different batch of HeLa cells and transcriptional effects by the use of aCGH, real-time PCR and whole transcriptome arrays.

All research works is carried out by means of conventional and molecular cytogenetic methods, and of molecular techniques, besides basic cell biology methods, as different cell culture techniques: the most relevant and informative methods used in relation to projects goals include Fluorescent In Situ Hybridization (FISH) and Multipainting, and microarray based comparative genomic hybridization (aCGH) or whole transcriptome microarrays; Quantitative real-time PCR for expression studies.

Teaching experiences

The teaching experiences (since 2009) includes:

- Course of General Biology and Medical Genetics in the Course for degree in Cardio-Circulatory Perfusion, at the University of Insubria
- Course of General Biology and Medical Genetics in the Course for degree in Dental Hygiene, at the University of Insubria
- Course of General Biology in the Course for degree in Laboratory Techniques, at the University of Insubria
- Course of General Biology in the Course for degree in Radiotherapy and Radiological Techniques, at the University of Insubria
- Course of Medical Genetics in the Course for degree in Obstetrics, at the University of Insubria
- Course of Medical Genetics in the Course for degree in Professional Education, at the University of Insubria
- Specific lessons of the course of General Biology and Medical Genetics in the Course for degree in Medicine and Surgery, at the University of Insubria.
4. Applicant’s Commitment as Investigator of the Project:

   I agree as the applicant to accept responsibility for the scientific management of this project as outlined in this application. I further agree to submit a report at the end of the granting period.

5. Applicant’s Affirmation:

   I certify that the investigations involving human subjects to be carried out in the application will have approval of the applicant’s Institutional Ethical Committee.

   Approvals from the Institutional Ethical Committee must be included with the application.

6. Research Results:

   Results of research may be made available to the public through appropriate scientific channels. All publications will bear the statement:

   “THIS WORK WAS SUPPORTED BY A GRANT FROM ASSOCIAZIONE ITALIANA SINDROME DI SHWACHMAN (AISS)”

   [Signature of Applicant]

   Varese, 02/03/2018
7. Applicant’s Institution Certification and Commitment:

I certify that the statements herein and the Applicant’s Affirmation are true, complete and accurate to the best of my knowledge and I agree to accept responsibility for the fiscal management of this project as outlined in this application. I further agree to commit this institution to comply with the Associazione Italiana Sindrome di Shwachman-Diamond (AISS) terms and conditions if a grant is awarded as a result of this application.

Name of Institution Official: Angelo Tagliabue, professor

Title: Director of the Department of Medicine and Surgery

Address:
Dipartimento di Medicina e Chirurgia
Via Guicciardini, 9
21100 VARESE

Phone: 0332-278830
Fax: 0332-217119

Email: angelo.tagliabue@uninsubria.it

Varese, 02/03/2018

Angelo Tagliabue
Director of Department of Medicine and Surgery
(signature)
ABSTRACT OF RESEARCH PLAN

Within the space provided, summarize the long-term objectives, scientific aims and methodology of the proposal.

TITLE: Deletions of the long arm of chromosome 20 in bone marrow: aCGH, expression analysis and development of a cellular model by the use of HL-60 cell line by the CRISPR/CAS9 system.

Scientific aims
The deletion of the long arm of the chromosome 20, del(20)(q), which is found in the bone marrow (BM) of many patients with Shwachman-Diamond syndrome (SDS) by cytogenetic monitoring, was shown to imply a benign prognostic role as to the development of myelodysplastic syndrome (MDS) and acute myeloid leukemia (AML). We performed array comparative genomic hybridization (aCGH) on DNA of BM from 12 patients carrying the del(20)(q). We have found the deletion of the EIF6 gene, located at 20q11.22 in all of them. The EIF6 gene is involved in the same pathway of SBDS gene: EIF6 protein must be removed from the ribosomal pre-60S subunit by the cooperation of SBDS and EFL1 proteins to allow the joining of the two ribosomal subunits and the initialization of the translation process. We postulated that EIF6 haploinsufficiency, due to the del(20)(q) deletion in SDS patients, could be related to a better “fitness” of the BM clone carrying the anomaly through a dosage mechanism.

The present project is focused on better investigating this mechanism of protection from MDS/AML by the use of routine cytogenetics, aCGH and whole genome expression analysis studied by array methods. This hypothesis will be tested and confirmed by the developing of a cellular model for SDS by modifying the well-known myeloid HL-60 cell line through the CRISPR/CAS9 system.

Methodology
We perform cytogenetic investigations since 1999 in a cohort of 96 Italian patients with SDS. Up to now, 18 patients have acquired a del(20)(q) in the BM. In 12 cases, it was possible to apply aCGH analysis and EIF6 gene was lost in all patients.

The material of the present project includes bone marrow specimens for routine and molecular cytogenetic analysis, and DNA and RNA from BM. The aCGH system which will be used is the 244K genome-wide system (Agilent Technologies Inc., Santa Clara, CA, USA) applied and analysed according to manufacturer’s instruction and software. Results will be validated by FISH with the probes indicated as informative by aCGH.

HL-60 cell line has been provided by the Banca Biologica e Cell Factory - IRCCS Azienda Ospedaliera Universitaria San Martino – Genova (Italy). Single pure clone of the cell line, carrying two chromosomes 7 and two chromosomes 20 will be purified by single-cell dilution. CRISPR/CAS9 sgRNA guide system will be designed by the MIT software (http://crispr.mit.edu/). sgRNAs, CAS9 enzyme and buffers will be purchased by New England Biolabs. Donor DNA ultramers will be manually designed to introduce the desired mutations and purchased by Integrated DNA Technologies (IDT) (https://eu.idtdna.com/pages/home).

Whole genome expression analysis by arrays will be performed on the RNA from BM of patients and on the HL-60 modified cell lines with the 8x60K human whole transcriptome array (Agilent Technologies Inc., Santa Clara, CA, USA) according to manufacturer’s instructions and data will be analysed by the use of R-software with suitable packages. A pool of genes which result to be up/down regulated will be validated by the use of standard real time PCR thermocycler ABI 7000 (Applied Biosystems, Foster City,
CA, USA) with suitable primers sets and SYBR green master-mix (Bio-Rad, Hercules, CA, USA). RNA from BM of healthy donors will be used as normal control both in transcriptome arrays as in real-time PCR experiments.

Long-term objectives

Up to now, we have identified 12 patients with SDS in whom the del(20)(q) always encompasses the \textit{EIF6} gene, at variance with the del(20)(q) in patients with MDS/AML in whom the deletion appears to be different from case to case and the loss of \textit{EIF6} not always present. The present project will focus on the following long-term objectives:

1 - We will follow-up the patients who carry the del(20)(q) clone to find out new cases to be evaluated, by routine cytogenetic monitoring.

2 – aCGH and FISH analysis will be performed in order to characterize precisely the deletion and to demonstrate the peculiar \textit{EIF6} loss already found in all other cases.

3 - RNA from BM of patients with \textit{EIF6} loss (demonstrated by aCGH and FISH) will be collected and whole transcriptome analysis by arrays will be performed. We expect to validate our preliminary transcriptome array data in three patients with the del(20)(q) at various clonal percentage. In these three patients, the dendrogram analysis of whole transcriptome and hematopoietic genes pathways showed that at higher percentage of del(20)(q), the overall expression pattern is more similar to normal donor subjects than in patients with low percentage of del(20)(q).

4 – We will develop a modified HL-60 cell line in which the two common \textit{SBDS} mutations (183-184TA>CT and 258+2T>C) will be introduced by the use of CRISPR/CAS9 genome editing system. This \textit{"SBDS_HL-60"} modified cell line will provide a cheap and useful model to further investigate the effects of the del(20)(q). To “mimic” the deletion encompassing \textit{EIF6} gene on the modified SDS_HL-60 cell line, we also plan to knock-out one allele of \textit{EIF6} gene again by the use of CRISPR/CAS9 system.

Whole transcriptome analysis by microarray will be performed on the SDS_HL-60 cell line and on SDS\textsubscript{EIF6}KO HL-60 cell line. We expect to have a restoring of the hematopoietic pathways in the cell line with the knockout of \textit{EIF6} gene as we observed in transcriptome analysis of the bone marrow of the patients with the del(20)(q) encompassing \textit{EIF6} gene.
BUDGET

List below a budget by categories for the support. The review committee will carefully consider the appropriateness of your budget. It must be well defined, justified, and realistic to complete the work proposed. The first column defines the total expenses that are expected to be necessary to realistically complete the project. The second column indicates the expenses requested from the AISS. Applicants will not be penalized in funding considerations for requiring additional funds beyond what is requested from the Foundation(AISS); however, the true costs of the project must be acknowledged. [This and the section on page 1 re: Other Funding need to be consistent]

EURO Amount Requested for:

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<th>TOTAL COSTS REQUIRED TO COMPLETE PROJECT:</th>
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<td>TOTAL COSTS:</td>
<td>€ 28,610</td>
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Justification: Define and justify expenses in each category. Explain the role of each of the individuals named in the Personnel section. The justification must include an explanation of what each category contributes to the project. Also explain any marked differences between the first- and second-year expenses in a particular category. The AISS will provide preference to those applications in which funds are used for supplies, equipment, technicians and other expenses and not for support of the salary of the PI or co-PIs. The AISS-SC may ask for further expense details.

Personnel and equipment for this research project are already available.
The entire group of researchers is involved in all parts of the research work.
The costs concern only supplies, and are in part covered by other grants already available.

- Each a-CGH test costs approximately € 670, including
  - Array platform
  - Labeling Kit
  - 244K aCGH array slides and Gasket slides
  - Washing solutions
  - Software

  No. aCGH test planned: 15 = 10,050

  Costs for cell cultures, conventional cytogenetics and FISH (when needed): approximatively € 300 per case

  Total foreseeable costs: 3,000

- Costs for real-time-PCR for the informative genes: approximatively € 100 per 96 well plate of real time

  Total foreseeable costs: € 2,000

- Whole transcriptome arrays: the format for this test is a multiple arrays. (8x60K) in which there are 8 cases for each array. The cost for each case/patient is approximatively € 220, including:
  - Array platform
  - Labeling Kit
  - 8x60K whole transcriptome arrays and gasket slides
  - Washing solution
  - Software

  No. of Whole transcriptome arrays planned: 48 = 10,560

- CRISPR/CAS9 on HL-60 cell line: Every CRISPR/CAS9 experiment have an estimated cost of approximatively € 1,000 including:
  - sgRNAs
  - CAS9
  - Buffers
  - IDT ultramrs
  - Culture’s mediums
- Disposables

No. of CRISPR/CAS9 experiment planned: 3 = 3,000

- Total supplies costs: € 28,610
Other Support for this Project:
Applicants are allowed to receive funding from other sources for parts of the project not funded by the AISS. Please, list all other funding sources.

- Università dell’Insubria FAR 2016 e 2017
Section 2

AISS Applicant: Roberto Valli

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Research Plan

Part A – Specific aims
The present project will focus on the interstitial deletion of the long arm of the chromosome 20, del(20)(q), by studying it in the bone marrow (BM) of the patients and by generating a suitable cellular model. The del(20)(q) is a change frequently found in the bone marrow (BM) of patients with Shwachman-Diamond syndrome (SDS) and it is considered a relatively benign prognostic sign due to the consequent loss of the *EIF6* gene (located at the band 20q11.22, in the deleted region). As the *EIF6* gene is involved in the same pathway of the *SBDS* gene, we previously observed that its haploinsufficiency (due to the del(20)(q) deletion) is related to a better "fitness" of the BM clone carrying the anomaly through a dosage mechanism. The results may be relevant for the study of pathogenic mechanisms leading to (or protecting from) Myelodysplastic syndrome (MDS) and Acute Myeloid Leukaemia (AML), with obvious practical implications for the best clinical management of the patients.

Specific aims of the present project are:
- a further investigation of the del(20)(q) and of its clinical role;
- a study of gene expression of BM cells bearing the deletion compared with BM cells of patients with SDS but without del(20)(q);
- the development of a cellular model with SBDS common mutations and with the knockout of the *EIF6* gene that mimic the del(20)(q) and that will be analysed in relation to BM function.

Part B – Significance and background

The proportion of patients with SDS who develop MDS and/or AML is not far from 30% (Dror, 2005), and evaluations which take account the age of the patients estimate a risk of 19% at 20 years and of 36% at 30 years (Shimamura, 2006). Clonal chromosome changes, mainly involving the chromosomes 7 and 20, are often found in BM of SDS patients. The most frequent are: an isochromosome of the long arm of chromosome 7, i(7)(q10), and the deletion of the long arm of chromosome 20, del(20)(q) (Maserati et al, 2006). Moreover, we observed the recurrence in SDS patients of other anomalies involving the chromosome 20 and/or other chromosomes by the use of aCGH (Valli et al, 2017). The relationship between these and other chromosome changes in the BM and the risk of MDS/AML is object of discussion (Dror et al, 2002; Dror, 2005). Since 1999 we collected a cohort of 96 Italian SDS patients that we routinely monitor by cytogenetics in order to early discover any chromosome acquired anomaly that could be related to a progression into MDS/AML. During these years, we showed that the acquisition of BM clonal chromosome anomalies is age-related, in parallel with the risk of MDS/AML (Maserati et al, 2009). We suggested a mutator effect of *SBDS* mutations, leading to a specific type of karyotype instability which leads, in turn, to the clonal anomalies in the BM (Maserati et al, 2006). This view was supported by the evidence that SBDS protein promotes spindle stability and normal chromosome segregation (Austin et al, 2008): the defect in SDS cells may so explain the karyotype instability, possible through cytokinesis failure and tetraploidy, with subsequent chromosome changes.

Comparative genomic hybridization on microarray (aCGH) provides a powerful tool to investigate unbalanced chromosome anomalies, and some results in SDS have been already reported (Maserati et al, 2009). The definition of aCGH is much higher than conventional cytogenetics and can detect cryptic or very small chromosome imbalances. In particular an exhaustive analysis by aCGH was performed on the BM of patients carrying the del(20)(q). We showed that 6/6 patients with the del(20)(q) invariably loose the *EIF6* gene, located at 20q11.22, although the deletion could be very different from patient to patient (4.14 Mb the smallest, 26.86 Mb the largest) (Valli et al, 2013). Up to now, 6/6 further new patients analysed showed the same loss of *EIF6* (unpublished data). So, the loss of *EIF6* seems to be present in every SDS patients with the del(20)(q). On the contrary, the del(20)(q) imbalance that occurs
rather frequently in patients with myeloproliferative neoplasms, may imply or not the loss of EIF6 (Valli et al, 2013).

It is intriguing that the EIF6 protein itself is the target of the SBDS/EFL1 GTPase action in the early ribosome assembly. It is necessary to remove EIF6 factor from the pre-60S subunit to let the formation of the 80S ribosome and to start the translation (Finch et al, 2011). SDS patients have very low levels of SBDS protein in the BM leading to an abnormal accumulation of the pre-60S subunit. So, SDS is considered a ribosomopathy, as, at the end, the overall translation of specific tissues (BM in primis) is compromised (Finch et al, 2011). It is therefore possible to hypothesize a dosage-dependant mechanism: the haploinsufficiency of EIF6 (due the del(20)(q) in many SDS patients) causes a lower dosage of EIF6 protein which, together with SBDS low levels due to the SBDS mutations, lead to a better ribosome assembly in the clone carrying the anomaly and, in fact, to a better "fitness" of the clone itself and to a lower risk to develop MDS/AML. (Pressato et al, 2012. Valli et al, 2013).

The first aim of this research project, in parallel with the standard long lasting cytogenetic analysis, is to perform aCGH analysis on all available patients with or without the del(20)(q) in order to verify the loss of EIF6 gene.

Little is known about the expression patterns of BM in SDS patients. Only Ruijikjyanont and colleagues (Ruijikjyanont et al, 2007 and 2009) performed whole transcriptome analysis of RNA from BM of SDS patients and normal donors, putting in evidence the activation of leukaemogenesis pathways in SDS patients and disruption of ribosome biogenesis and RNA processing. Our group has conducted a preliminary whole transcriptome study (Valli et al, 2016) on three patients carrying the del(20)(q) and we found a clear direct correlation between the amount of cells with del(20)(q), in term of clonal percentage in BM, and the evidence of "restoring" a normal expression pattern. In the years, we collected RNA from bone marrow of 14 further different patients, 4 of them carrying the del(20)(q) at different clonal percentages. So, the second aim of this project is to perform a larger whole transcriptome study to confirm the preliminary experiment described above with a more robust statistical approach, with a comparison with the BM of patients without the del(20)(q) and with normal control subjects.

Third aim: to verify further the hypothesis of EIF6 haploinsufficiency as related to a better prognosis, we plan to generate a cellular model to investigate the effects of EIF6 haploinsufficiency. Our strategy is to use the well-known HL-60 myeloid cell line and to introduce the SBDS 183_184TA>CT and 235+2T>C mutations with the help of the brand-new technology CRISPR/CAS9. Once obtained a pure HL-60 clone carrying the two common SBDS mutations in SDS patients, we plan to knockout one allele of EIF6 again with the help of CRISPR/CAS9 technology. Finally, with the help of whole transcriptome arrays, we will verify if the loss of EIF6 (in a cellular model in which there are the two mutations of SBDS common to patients) might lead to restore the transcriptome pattern as observed in RNA from bone marrow of patients with the del(20)(q).

Part C – Preliminary studies

1) Up to now, by long lasting cytogenetic monitoring of all the patients of our cohort, we have found 18/96 SDS patients carrying del(20)(q). In particular:
   - 10 of them have been characterised by aCGH, as the clonal amount of abnormal cells was sufficient (5 already reported in Valli et al, 2013)
   - 2 of them had a very small cryptic del(20)(q) only detectable by aCGH (one already reported in Valli et al, 2013)

2) We collected the RNA from BM of three patients with del(20)(q) with different clonal percentage of abnormal cells and two patients without the del(20)(q). We performed whole transcriptome array
analysis comparing these subjects with RNA from BM of 5 normal control donors. These preliminary results were presented at the 8th International Shwachman-Diamond Conference in Verona (Valli et al, 2016). Our results are of interest: in these three patients the dendrogram analysis of whole transcriptome and haematopoietic genes showed that at higher percentage of del(20)(q), the overall expression pattern is more similar to the normal donors subjects than in patients with low percentage of del(20)(q), prompting to a sort of “rescue” of the ribosome assembly in the clones carrying the anomaly that, eventually, restore the expression patterns.

In the last years we enlarged our cohort of specimens with RNA from bone marrow of other 14 different patients, 4 of whom carrying the del(20)(q) at various clonal percentages, and RNA from bone marrow of 4 other normal subjects as controls. We will replicate the same experiment as above to enlarge the cohort of patients analysed.

Part D – Experimental design and methods

The patients object of the study will be the ones of our cohort already under study, and all the SDS patients who will be detected during the development of the project, with or without the del(20)(q). Control subjects will be selected among healthy BM donors for transplantation. An informed consent will be obtained by the patients or the patients’ parents, as well as by the control subjects.

The material for the project includes BM and peripheral blood in heparin or EDTA, for cytogenetic or molecular analysis respectively. BM samples will be collected also by the use of PAX gene tubes (Qiagen, Hilden, Germany) in order to stabilise RNA. DNA and RNA will be extracted by Qiagen columns kits. All informative techniques of cytogenetics and of molecular cytogenetics will be used, in particular Fluorescent In Situ Hybridization (FISH) with informative libraries and probes, with the same methods and probes already described in previous publications (Maserati et al, 2006; Maserati et al, 2009; Pressato et al, 2012; Valli et al, 2013). The aCGH system used will be the 244K genome-wide system (Agilent Technologies Inc., Santa Clara, CA, USA), applied and analysed according to the manufacturer’s instruction and software. All aCGH results will be confirmed, when necessary, by FISH with the probes indicated as informative by the aCGH itself. Possible unknown benign Copy Number Variations will be confirmed on the peripheral blood of the patients and of their parents.

HL-60 cell line has already been obtained from the Banca Biologica e Cell Factory - IRCCS Azienda Ospedaliera Universitaria San Martino – Genova (Italy). Single pure clone of the cell line, carrying two chromosomes 7 and two chromosomes 20 will be purified by single-cell dilution. Small-Guide RNAs (sgRNAs) for performing CRISPR/CAS9 approach will be selected by the bioinformatic tool from Massachusetts Institute of Technologies (MIT) (http://crispr.mit.edu/). CAS9 enzyme and buffers will be purchased by New England Biolabs. Donor DNA ultramers will be manually designed to introduce the desired mutations and purchased by Integrated DNA Technologies (IDT) (https://eu.idtdna.com/pages/home).

Whole transcriptome analysis will be performed on the RNA from BM of patients and/or HL-60 cell lines with the 8x60K human whole transcriptome array (Agilent Technologies Inc., Santa Clara, CA, USA) according to manufacturer’s instructions. Data will be analysed by the use of R-software with suitable packages. EIF6 expression levels, as well as informative pools of up/down regulated genes, will be validated by the use of standard real time PCR thermocycler ABI 7000 (Applied Biosystems, Foster City, CA, USA) with suitable primers sets and SYBR green master-mix (Bio-Rad, Hercules, CA, USA). RNA from BM of normal donors will be used as normal control both in transcriptome arrays as in real-time PCR experiments.

Part E – References


Part F. Relevance of the research to Shwachman_Diamond Syndrome

The results of the research will be both of theoretical and practical relevance. In particular:

1. The results of aCGH will have a pivotal role in the precise definition of acquired unbalanced chromosome anomalies detected by chromosome analysis and FISH, especially for the cases that carry the del(20)(q) that will be further analysed by the use of whole transcriptome arrays
and real-time PCR.

2. Cryptic unbalanced chromosome anomalies, undetected by conventional cytogenetics, may be found by aCGH (as was in two patients of our cohort already reported in the literature and in a new case mentioned above) and should be discussed especially in relationship with the possible cryptic deletion of the chromosome 20 involving the loss of the EIF6 gene.

3. aCGH tool will have significant relevance (integrated with routine cytogenetics) in the precise detection of clonal anomalies (del(20)(q) in primis) and possible pathogenetic significance will be analysed.

4. Whole transcriptome analysis and the expression studies both in SDS patients (with and without the del(20)(q) and loss of EIF6) and in HL-60 modified cell lines, will shed light on pathogenetic mechanisms in SDS related to benign prognosis in general, and as to progression towards MDS/AML or severe anaemia.

Parts G, K
Not applicable