Possible Role of Pandemic AH1N1 Swine Flu Virus in a Childhood Leukemia Cluster

Supplementary Information

METHODS

Patients' biological samples

The bone marrow and peripheral blood samples were obtained with informed consent in accordance with the Declaration of Helsinki and with local ethical committee approval.

DNA extraction

Mononuclear cells were separated from bone marrow using Ficoll density gradient. DNA was extracted from mononuclear cells using the Wizard Genomic DNA Purification kit (Promega, Madison, WI, USA).

DNA index

DNA index was performed by flow-cytometry according to the guidelines provided by the Committee on Nomenclature of the Society for Analytical Cytology [1]. A DNA index value of 1 was considered as a measure of diploidy.

Cytogenetics

Chromosome analysis from bone marrow cells was performed according to standard laboratory techniques. Definition of a clone and the karyotypes were described according to ISCN 2013 [2].

PCR detection of risk polymorphisms

Genotyping of polymorphisms was carried out using allelic discrimination method by the TaqMan SNP Genotyping Assays. The following SNPs were tested: ARID5B gene (rs7089424), assay ID: C_29243783; CEBPE gene (rs2239633), assay ID: C_335486; IKZF1 gene (rs 4132601), assay ID: C_26019772; (Applied Biosystems, Life Technologies, Foster, CA, USA) [3-5].

PCR detection of minimal residual disease

The identification of the markers for MRD evaluation have been performed by PCR and heteroduplex analysis. Junctional regions of monoclonal PCR products were sequenced, and patient-specific junctional region sequences of PCR targets were identified. Allele-specific oligonucleotide (ASO) primers were designed complementary to the junctional region sequence of each target. PCR targets were tested for specificity and sensitivity. Real-Time Quantitative PCR (RQ-PCR) analysis was performed and interpreted according to the guidelines developed within the "European Study Group for MRD detection in ALL" (ESG-MRD ALL) [6,7].

Reverse-Transcription Polymerase Chain Reaction (RT-PCR).

cDNA was synthesized from 1μg total RNA in 20 μl total volume by using random hexamers. RT-PCR for ETV6-RUNX1, BCR-ABL1, MLL-AFF1(AF4), and TCF3-PBX1 fusion genes was performed as previously described [8].

Copy number and LOH analysis

High quality genomic DNA at diagnosis was analyzed with Affymetrix® Cytogenetics Whole-Genome 2.7M Array (Affymetrix, Inc., Santa Clara, CA, USA). Basically, 100 ng (33 ng/μl) of genomic DNA were processed following the Affymetrix® Cytogenetics Assay Protocol. The Affymetrix® Cytogenetics Reagent Kit was used to amplify, fragment, label and then denature the genomic DNA prior to hybridization onto the arrays. After hybridization the chips were than washed, stained (streptavidin-PE) and scanned using the Gene Chip® Scanner 3000 7G.

CEL files generated by the GeneChip Scanner 3000 7G were analyzed using the Chromosome Analysis Suite 1.1.0 software (Affymetrix) based on hg19 assembly.

For copy number aberrations detection the following filters were applied: amplifications ≥ 50kb and deletions ≥ 30kb, containing a minimum of 20 markers in the region, were considered as significant. Detection of long continuous stretches of homozygosity (LCSH) and copy number neutral loss of heterozygosity (LOH) was limited to aberrations longer than 2000 kb. Germline copy number variations (CNVs) and LCSH were defined as all abnormalities found in both the diagnosis and remission samples.

Data are in Supplementary Table 1.

Antibody test for influenza AH1N1 virus

Serum from pre-vaccination blood samples of 192 children aged from six months to nine years was collected and cryopreserved in September 2010 for a study on vaccination.

Anti-influenza *AH1N1* antibody titres were measured by the use of hemagglutination (HA)-inhibition assay, performed in accordance with established procedures [9-11]. Serum HA-inhibition antibodies were titred by a standard microtitre method; sera were previously treated with receptor destroying enzyme to remove non-specific HA-inhibitors. For each individual, serum samples collected were tested simultaneously in doubling dilutions from 1:10 to 1:2560. To increase the sensitivity of the test, antigens prepared from infected allantoic fluids were previously treated with Tween-ether [10]. For the purpose of this study, titres ≥ 1:40 were considered to reduce the risk of clinical infection with pandemic (H1N1) 2009 influenza by 50% [12].

Dried whole blood smears (DBS) from diagnostic samples of the patients with ALL (and controls) were also analyzed. Dried blood was scraped from each smear, soaked in 250 μL of elution buffer (phosphate buffered saline containing Tween 20 and 5% non-fat dry milk) and incubated for 30-60 min at room temperature. Samples were then centrifuged at room temperature (30 min at 1800×g) and eluate stored at -20°C until tested with the same method as for the serum [13,14].

Statistical analyses

ALL cases in Milan

The case series 1999-2000 defined as 0-14 year old children diagnosed with ALL resident in Milan at the time of diagnosis was taken from the Cancer Registry of Milan for the time period from 1999 to 2006 and from the hospital discharge forms from 2007 to 2010. Completeness and accuracy of clinical description of incident cases from the latter were checked with the National Childhood Cancer Registry.

Number of ALL cases in Milan was compared to the expected one on basis of the pooled lymphoid leukemia incidence rate of North West of Italy from the Italian Association of Cancer Registries [15]. The ALL incidence rate was not available, but it is known that ALL cases represent 99.2% of all the lymphoid leukemia cases [16].

Data are in Supplementary Table 2.

Global spatial heterogeneity

For testing the existence of a global spatial heterogeneity in the incidence of childhood ALL in Milan, we considered two methods. The first one was the Potthoff-Whittinghill method [17] that assumes no spatial heterogeneity among administrative areas under the null hypothesis (number of cases in each area is Poisson distributed) and over-dispersion under the alternative hypothesis (number of cases in each area follows a negative binomial distribution). The second method was the Moran index [18] that evaluates an autocorrelation index between the incidence rates in the administrative areas, considering three different time intervals (1999-2010; 1999-2006; 2007-2009).

Comprehensive investigation plan

The Healthy Regional Agency developed a comprehensive investigation plan aimed to ascertain the association of any of the postulated risk factors of childhood leukaemia with the observed cluster. The plan included:

i. a structured interview administered to both parents of each case by a physician epidemiologist (SD) *ad hoc* trained to use a validated questionnaire developed for a parallel study of the 'Italian Multicentric Study on Etiology of Childhood Lympho-Hemopoietic Tumors and Neuroblastoma (SETIL) [19].

ii. environmental analyses of chemical and physical agents, paying particular attention both to the schools and the residential environment.

A composite socio-economical status (SES) score for cases and controls was computed combining educational level of both parents with the median income of population living in the same census area (no more than 600 people).

Reproductive history of the mothers and familiarity for relevant diseases

The occurrence of pregnancies (number, type and mother's age at the event), miscarriages, stillbirths and hormonal treatments for contraception, ovarian stimulation and assisted reproduction were registered. Similarly, the occurrence of malignancies and other major diseases among first grade relatives of the parents was unveiled by submitting to them a list of common definitions of the relevant diseases.

History of exposure to physical and chemical risk factors

Pre-conceptional and *in utero* exposure of each case to ionizing radiation was assessed questioning about maternal and paternal exposure to occupational as well as diagnostic and therapeutic X-rays in the six months antecedent conception, as well as maternal exposure during pregnancy. Lifetime child exposure to diagnostic and therapeutic X-rays was ascertained via parent interview and, when necessary, examining the relevant clinical charts.

In utero exposure to ELF and radio frequencies was assessed questioning on the frequency and duration of maternal use of domestic/occupational electric appliances and cell phones during pregnancy. Direct child exposure to ELF and radio frequencies was also assessed by parents' interview.

In utero and lifetime exposure to toxic chemicals was sought, asking parents about their occupational and leisure activities (painting, gardening, modelling, etc.) and smoking habits. Domestic, garden and pets insecticides, as well as lice treatment compounds were also questioned.

A specific set of other variables including birth order, infectious episodes in the first year of life, age at the first day care attendance, pets exposure, vaccination against seasonal influenza and/or anti AH1N1 in the months preceding ALL diagnosis was also analyzed.

A comparison was performed with a control group consisting of parents of children matched to cases by sex and age (three month period) and attending three schools in the same area of Milan.

Environmental analyses of chemical and physical agents

A comprehensive investigation on the current concentrations of physical (ionizing radiations, ELF and radio frequencies magnetic fields) and chemical (polycyclic aromatic hydrocarbons, volatile organic compounds, pesticides and formaldehyde) agents in the dwellings and schools environment of the cases was performed.

Measurements were taken in the classes of the cases, in a sample of the other classes, in the common areas of the schools and in the three rooms of the living houses where each case used to spend most time:

ionizing radiations

- gamma dose intensity, beta-gamma ratio and indoor radon concentration.

- alpha and beta activity in the drinkable water sampled at school and dwelling taps.

non ionizing radiations

- intensities of magnetic field induction at 50 Hz with high and low electric load and at radio frequencies.

toxic chemicals

- air concentration of polycyclic aromatic hydrocarbons (PAH): benzo (a) anthracene, benzo (b) fluoranthene, benzo (a) pyrene, benzo (j) fluoranthene, benzo (k) fluoranthene, dibenzo (ah) anthracene, indeno (1,2,3 (cd) pyrene).

- air concentration of volatile organic compounds (VOC): benzene, ethylbenzene, tholuene, xylene, styrene, formaldehyde.

- physico-chemical characteristics of drinking water sampled at schools and houses.

- concentration in the air of dioxins and polychlorinated biphenyls (PCBs) in the home of one of the cases, in which there was a fire in the summer of 2008.

- presence of plant protection in the food served by the school canteen.

The technical specifications of each survey are available from the authors on request.

Other proxies of physical and chemical agents exposure in the dwelling and in the schools environment of the cases

Other possible sources of exposure to chemical and physical agents have been verified via:

- Verification of the presence of radioactivity from the use of sources of ionizing radiation for medical, industrial and veterinary use located in rooms adjacent to the schools and housing.

- Presence of power lines in air and underground within a radius of 200 m from schools and homes.

- Presence of radiofrequency antennas such as radio, television and mobile phone within a radius of 500 m from schools and homes.

- Verification of the technical characteristics of the furniture and cleaning products used in schools.

- Reviewing the data sheets of substances used for pest control activities in schools.

- Examination of the technical specifications of the material used for the renovation of the school.

Main results of the Comprehensive Investigation Plan

Patients

The main biological features of the patients at disease presentation are shown in Supplementary Table 1. All were 'common' B cell precursor (CD10 positive) ALL, five female and two male; the median age was 7.1 (range 2.1 – 11.9), the median WBC count was 9030/ul (range 960 – 48760). One patient carried the t(9;22) translocation, one was t(12;21) positive, three were hyperdiploid (more than 50 chromosomes), while one carried a minor hyperdiploid clone and the latter was pseudodiploid. Copy number analyses found deletions in some of the genes related to B-cell differentiation frequently altered in B-cell precursor ALL [20], although none of them was recurrent in more than two cases (two patients carried *CDKN2A* deletion and two *IKZF1* deletion, in one of them associated to iAMP21). Gene polymorphisms associated with an increased risk to develop ALL were analyzed [3-5], and only one case carried more than four risk alleles, thus showing an increased odd ratio.

In summary, all seven patients including the three patients from the same school had the spectrum of biological and clinical features typical of childhood common ALL (Supplementary Table 1).

Physical and chemical agents in the residence and in the schools of the cases

The exposures to all tested physical and chemical agents in the school as well as in the houses of cases did not deviate from normal ranges, with the exception of the vicinity to electricity cables in the entrance of the school: 2.3 μT at the floor and 0.4 μT at 1 meter high (although below the attention limit of 10 μT, and far below the bylaw exposure limit of 100 μT).

Neither parents nor children cases had professional or therapeutic exposures; all but the youngest children received one to three radiographies in the previous years.

None of the tested chemicals showed values exceeding the normal ranges. Three of the seven fathers had professional exposures (constructions or dental studio), more likely very low, although not quantifiable.

There was no evidence for carcinogenic or mutagenic material used for cleaning, disinfecting or reconstructing the school.

Tumor risk in the families

None of the cases had first level familial malignancies. One parent and one to three grand-parents in all seven cases were affected by cancer, with one family having several cases of breast cancer, and one of the cases had a brother with type 1 neurofibromatosis.

With the exception of one family, at least one of the parents was or has been smoking, including in the last six months before pregnancy, but mothers did not smoke during pregnancy.

The full Report of the Comprehensive Investigation Plan is publicly available (in Italian) at the web site http://www.asl.milano.it/user/download.aspx?FILE=OBJ02043.PDF&TIPO=FLE&NOME=report_giugno_2010

Specific information will be provided upon request.

Global spatial heterogeneity

We did not observe any difference in incidence rates when analyzing the spatial over-dispersion among areas of the city, both with Potthoff-Whittinghill's test and Moran index (Supplementary Table 3). We did not observe spatial over-dispersion among areas of the city, both in the whole time interval (1999-2009) and in the sub-periods (1999-2006, 2007-2009): none of the p-value of the tests was significant. Neither the analysis of spatial autocorrelation did evidence any spatial heterogeneity among incidence rates in the administrative areas.

Space-time cluster analysis

A Scan statistic for the space-time analysis of ALL cases has been performed (Supplementary Table 4 and Figure 2 in main text). The Kulldorff's scan method identified only one significant cluster encompassing six administrative areas in the center of Milan within the time interval from 1 December 2009 to 31 January 2010 (p-value = 0.017). The cluster is composed of four cases, compared to 0.04 expected, corresponding to a rate ratio of 100.0 (95%CI: 26.9-256.0) for the areas and time period identified. The cases are exactly the four children resident in the surroundings of the school, three of them attending the school. Space-time analysis identified other clusters in the time period 1999-2009, but the corresponding p-values were not significant. In Figure 2 only the most likely clusters (p–value < 0.9) are shown.

AH1N1 antibody screens

Supplementary Table 5 summarizes the AH1N1 antibody titre data.

Social contacts and socio-economic status of cases and controls

A composite socio-economical status (SES) score for cases and controls was computed combining educational level of both parents with the median income of population living in the same census area (no more than 600 people) (Supplementary Table 6).

Controls were sex and age matched to cases and taken from the school used temporarily by the cases when their school was being refurbished (see Figure 2 in main text).

Supplementary Table 1.

Biological features of the patients at diagnosis.

UPN	Date (week/y)	Age (y)	Sex	WBC ($x10^6$/L)	Fusion transcripts				DNA index	Cytogenetics	SNP arrays°	Risk alleles*			
					t(4;11)	t(9;22)	t(12;21)	t(1;19)				ARID5B (rs7089424)	CEBPE (rs2239633)	IKZF1 (rs4132601)	Total number of risk alleles
3593[1]	51/2009	8·3	F	48,760	neg	**pos**	neg	neg	1.00	48,XX,t(9;22)(q34;q11),+2 mar[7]	del(IKZF1), iamp21	A**C**	**GG**	A**C**	4
3597	51/2009	11·9	M	1,400	neg	neg	neg	neg	1.16 (20%)	NA	del(CDKN2A/2B/MTAP)	A**C**	**GG**	A**C**	4
3602	51/2009	5·8	F	4,710	neg	neg	neg	neg	N.A.	Hyperdiploid, 53 chromosomes	+4,+6,+10,+14,+18,+21,+21,+X, del(IKZF1)	AA	**GG**	A**C**	3
3616[1]	1/2010	7·1	F	960	neg	neg	neg	neg	N.A.	NA	+6,+8,+10,+14,+15,+17,+21,+21,+X	A**C**	A**G**	AA	2
3629	2/2010	6·5	F	9,030	neg	neg	**pos**	neg	1.00	NA	del(CD200), del(CDKN2A/2B/MTAP), del(RB1), del(RUNX1), +X	GG	A**G**	AA	1
3630[2]	2/2010	2·1	F	9,200	neg	neg	neg	neg	1.17	55-57,XX,+4,+6,-8,-9,+10,+15,+15?,+17,-17,+21,+21,+22,+22,+8mar [cp4]/46,XX[11]	+1q,+4,+6,-8p,+10,+14,+21,+21,+21,+22,+X	**CC**	**GG**	A**C**	5
3635[1]	2/2010	10·9	M	11,610	neg	neg	neg	neg	pseudodiploid	NA	none	AA	**GG**	AA	2

UPN=Unique Personal Number; [1] children from the same school; [2] sister of a non affected child from the same school; ° main genomic aberrations as detected by SNParrays are indicated; * according to Prasad RB et al. (5)

Supplementary Table 2.

Number of cases of acute lymphoblastic leukemia (0-14 years of age) observed in Milan in the time period 1999-2009.

Year of incidence	Observed cases[1]	Population 0-14[2]	Incidence rate[3]	Expected cases[4]	O/E ratio (95% CI)
1999	7	139325	50·24	6·27	1·12 (0·51-2·46)
2000	7	141937	49·32	6·39	1·10 (0·50-2·42)
2001	8	140885	56·78	6·34	1·26 (0·60-2·65)
2002	9	145122	62·02	6·53	1·38 (0·68-2·77)
2003	8	146674	54·54	6·60	1·21 (0·58-2·55)
2004	5	150252	33·28	6·76	0·74 (0·29-1·89)
2005	6	155120	38·68	6·98	0·86 (0·36-2·03)
2006	8	157616	50·76	7·09	1·13 (0·54-2·38)
2007	6	158632	37·82	7·14	0·84 (0·36-1·99)
2008	9	160323	56·14	7·21	1·25 (0·62-2·52)
2009[5]	12	163836	73·24	7·37	1·63 (0·88-3·00)

Population resident in Milan (0-14 years of age), annual incidence rate, number of expected cases, and observed:expected ratio (O/E ratio) with corresponding 95% confidence interval (95% CI) are indicated.

[1]from Cancer Registry of Milan 1999 – 2006; verified hospital discharged data 2007–2010.

[2]data from the municipality of Milan.

[3]over 1·000·000 person/year.

[4]estimates based on the pooled incidence rate of North Italy (1998-2008) from the Italian Association of Cancer Registries (AIRTum).

[5]observed cases in 2009 include 3 cases from the cluster in object, diagnosed in December.

Supplementary Table 3.

Number of acute lymphoblastic leukemia cases and results from Potthoff-Whittinghill's test and Moran index.

Temporal interval	Number of cases	Potthoff-Whittinghill's test p-value	Moran's index p-value
1999-2009	85	0·26	0·46
1999-2006	58	0·10	0·62
2007-2009	27	0·20	0·67

Results are provided according to time interval considered.

We did not observe spatial over-dispersion among areas of the city, both in the whole time interval (1999-2009) and in the sub-periods (1999-2006, 2007-2009): none of the p-value of the tests was significant. Neither the analysis of spatial autocorrelation did evidence any spatial heterogeneity among incidence rates in the administrative areas.

Supplementary Table 4.

Scan statistic for space-time analysis of ALL cases, considering monthly time interval. Milan, 1999-2010

Areas	Time interval	Observed cases	Expected cases	p-value
Cluster 1	01/12/2009 – 31/01/2010	4	0·04	0·017
Cluster 2	01/12/2000 – 31/10/2001	6	0·46	0·586
Cluster 3	01/12/2000 – 31/10/2001	5		
Cluster 4	01/05/2002 – 30/09/2002	2	0·02	0·993
Cluster 5	01/07/2005 – 28/02/2006	2		

The applied model does not include the non parametric adjustment for temporal trend.

Supplementary Table 5.

Analysis of H1N1 infection.

Group	Pt.	date of diagnosis (week/y)	age	sex	diagnosis	Ab H1N1 plasma PB	Ab H1N1 PB smear
Cluster	1	51/2009	8·3	F	ALL		160
	2	51/2009	11·9	M	ALL		320
	3	51/2009	5·8	F	ALL		320
	4	01/2010	6·5	F	ALL		160
	5	02/2010	5·6	F	ALL		320
	6	02/2010	2·1	F	ALL		640
	7	02/2010	10·9	M	ALL		640
post-H1N1	8	30/2010	4·2	M	ALL		40
	9	46/2010	3·1	M	ALL	1280	160
	10	46/2010	4·0	M	ALL	80	40
	11	01/2011	15	M	ALL		40
	12	03/2011	1·2	M	ALL	160	80
pre-H1N1	13	15/2008	5·4	M	ALL		<10
	14	18/2008	8·5	M	ALL		<10
	15	23/2008	2·6	M	ALL		<10
	16	24/2009	14·3	M	ALL		<10
	17	32/2008	6·8	M	ALL		<10
solid tumors	18	4/2011	3·2	M	WT	1280	640
	19	50/2010	3·9	M	WT	<10	<10
	20	46/2010	9·0	F	WT	<10	<10
	21	48/2010	1·5	M	WT	<10	<10

WT: Wilms tumor cases, Lombardy Region.

Supplementary Table 6.

Social-socio-economic features of cases and controls.

		Cases (#7)	Controls (#20)
1.	Birth order: 1st born	6	9/14*
2.	Day care attendance – 1st year	0	2
3.	Day care attendance – anytime (- 3 years)	2	8/14*
4.	Socio-economic status: High Medium-high Medium Medium-low Low	 5 2 0 0 0	 0 2 10 2 6

* for controls the information is missing.

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