INTRODUCTION

ACUTE MYELOID LEUKEMIA

DEFINITION

Acute myeloid leukemia (AML) is a heterogeneous clonal stem cell malignancy in which immature hematopoietic cells proliferate and accumulate in bone marrow, peripheral blood, and other tissues. This process results in inhibition of normal hematopoiesis, and the clinical features of bone marrow failure. AML accounts for 90% of all acute leukemias in adults, with approximately 13,000 new cases and 9,000 deaths in the United States in 2009. The annual incidence is approximately 3.5 per 100,000 and increases with age, with approximately a tenfold increased risk between ages 30 (1 case per 100,000) and 65 years (1 case per 10,000). The median age at diagnosis is 67 years, with ~6% of patients <20 years of age and 34% of patients 75 years or older. Overall survival in adults remains poor, with <50% 5-year survival in patients <45 years of age and <5% in patients >65 years of age at diagnosis. In children, overall survival has improved to ~60%. [1]

Most cases of AML have no apparent cause. The most common known risk factor is previous exposure to radiation or chemotherapy, particularly topoisomerase II inhibitors and alkylating agents, which results in therapy-related AML (t-AML), and accounts for ~10%-20% of all AML cases. Those that arise after exposure to alkylating agents or radiation therapy have increased incidence with age, typically have a 5- to 10-year latency period, and frequently are associated with an antecedent therapy-related myelodysplastic syndrome (MDS) and unbalanced loss of genetic material involving chromosomes 5 or 7. t-AML associated with exposure to topoisomerase II inhibitors encompasses 20%-30% of t-AML patients, has a shorter latency period of 1-5 years, is less often preceded by a myelodysplastic phase, and may be associated with balanced recurrent chromosomal translocations involving 11q23 (MLL) or 21q22 (RUNX1). Other environmental risk factors include exposure to benzene and ionizing radiation. Patients with inherited bone marrow failure syndromes (eg, Fanconi anemia, Shwachman-Diamond syndrome, severe congenital neutropenia), genetic disorders (eg, Down syndrome), and MDS and myeloproliferative disorders are also at increased risk of developing AML and have poor treatment outcomes. [1]

CLASSIFICATION OF AML

In the 1970s, AML was subclassified according to the French- American-British (FAB) classification system using morphologic and cytochemical criteria to define eight major AML subtypes (M0-M7). The FAB system has been largely replaced by the World Health Organization (WHO) classification, which was developed to incorporate biology, immunophenotype, and genetics into the diagnostic criteria. AML is now defined as greater than or equal to 20% myeloblasts, monoblasts or promonocytes, erythroblasts, or megakaryoblasts in the peripheral blood or bone marrow, except in patients with the following cytogenetic abnormalities, who are classified as having AML irrespective of blast count: t(8;21)(q22;q22), inv(16)(p13q22), t(16;16)(p13;q22), and t(15;17)(q22;q12). Immunophenotypic characterization using surface antigens remains important in AML and may include progenitor-associated antigens (eg, human leukocyte antigen-DR [HLA-DR] [except in APL], CD34, CD117) and myeloid antigens (eg, CD13, CD33); complex composite immunophenotypes, including non-lineage-restricted lymphoid markers, also may be seen. [1]

The world health organization (WHO) classification takes into account molecular genetics, therapy – related leukemias, and biphenotypic leukemias:

I. AML with recurrent genetic abnormalities

- AML with t(8; 21) (q22; q22); RUNX1-RUNX1T1
- Acute promyelocytic leukemia with t(15; 17) (q22; q12); PML-RARA
- AML with inv (16) (p13; q22) or t(16;16)(p13;q22); CBFB-MYH11 with abnormal marrow eosinophils
- AML with 11q23 (MLL) abnormalities

II. AML with multilineage dysplasia

- With prior myelodysplastic syndrome (MDS)
- Without prior myelodysplastic syndrome

III. AML and MDS: therapy related

- Alkylating agent related
- Epipodophyllotoxin- related
- Others.

IV. AML otherwise not categorized

- AML minimally differentiated
- AML with maturation
- AML without maturation
- Acute myelomonocytic leukemia
- Acute monocytic leukemia
- Acute erythroid leukemia
- Acute megakaryocytic leukemia
- Acute basophilic leukemia
- Acute panmyelosis with fibrosis [2, 3]

V. Acute biphenotypic leukemias

The WHO classification of AML continues to evolve and incorporate newly detected molecular mutations with a defined impact on disease biology. The hope is that continuing refinement of the classification into biologically distinct entities will define prognosis and help guide the type and intensity of therapy. Ultimately, better understanding of the signaling pathways in leukemogenesis will lead to a more sophisticated subclassification into disease entities, especially for sensitivity to different targeted therapies.[4]

ETIOLOGIC AGENTS AND ASSOCIATIONS OF AML

Although several factors have been implicated in the causation of AML, most patients who present with de novo AML have no identifiable risk factor. [5]

1-Antecedent hematologic disorders:

The most common risk factor for AML is the presence of an antecedent hematologic disorder, the most common of which is MDS. Other antecedent hematologic disorders that predispose patients to AML include paroxysmal nocturnal hemoglobinuria, and myloproliferative neoplasms. [3]

2-Congenital disorders:

Some congenital disorders that predispose patients to AML include Bloom's syndrome, Down syndrome, Fanconi anemia, and neurofibromatosis. More subtle genetic disorders, including polymorphisms of enzymes that metabolize carcinogens, also predispose patients to AML. For example, polymorphisms of glutathione *S*-transferase are associated with AML following chemotherapy for other malignancies.[3]

3-Familial syndromes:

Mutation of *CEBPA* (the gene encoding CCAAT/enhancer binding protein, alpha; was described in a family with 3 members affected by AML. Some hereditary cancer syndromes, such as Li-Fraumeni syndrome, can manifest as leukemia. However, cases of leukemia are less common than the solid tumors that generally characterize these syndromes. [4]

4-Environmental exposures:

Several studies demonstrate a relationship between radiation exposure and leukemia. Patients receiving therapeutic irradiation for ankylosing spondylitis were at increased risk of leukemia. Survivors of the atomic bomb explosions in Japan were at a markedly increased risk for the development of leukemia. [4]

Persons who smoke have a small but statistically significant increased risk of developing AML. Exposure to benzene is associated with aplastic anemia and

pancytopenia. These patients often develop AML. Many of these patients demonstrate M6 morphology. [4]

5-Previous exposure to chemotherapeutic agents:

Patients with previous exposure to chemotherapeutic agents can be divided into 2 groups:

(1) Those with previous exposure to alkylating agents

Patients with a previous exposure to alkylating agents, with or without radiation, often have a myelodysplastic phase before the development of AML. Cytogenetics testing frequently reveals -5 and/or -7 (5q- or monosomy 7).

(2) Those with exposure to topoisomerase-II inhibitors.

Patients with a previous exposure to topoisomerase-II inhibitors do not have a myelodysplastic phase. Cytogenetics testing reveals a translocation that involves chromosome band 11q23.

The typical latency period between drug exposure and acute leukemia is approximately 3-5 years for alkylating agents/radiation exposure, but it is only 9-12 months for topoisomerase inhibitors. [4]

MOLECULAR BIOLOGY OF AML

At the molecular level, pathogenesis of AML is a complex multistep process that results from the interaction of two different classes of mutations.[4, 6] The first class of mutation blocks cell differentiation resulting in clonal expansion of myeloid progenitors. The second class causes abnormal cell proliferation by constitutive activation of cellular proto-oncogenes including FLT3 tyrosine kinase, RAS, c-KIT, and others. The silencing of tumor suppressor genes also contributes to the pathogenesis. Some molecular genetic alternations in AML are highlighted by distinct chromosomal changes including translocations, inversions, and deletions while others can be only identified by molecular analysis.[7] The analysis of these cytogenetic and molecular changes is used to predict clinical outcomes and to formulate treatment paradigms in AML. Studies are underway to understand the downstream molecular pathways triggered by these mutations that lead to the unrestricted growth of leukemic cells and suppression of normal hematopoiesis by the malignant clone.[4]

Table 1: Common Cytogenetic Abnormalities And Mutations In Acute Myeloid Leukemia

Mutations that block differentiation	**Mutations that promote proliferation**
Balanced translocations and inversions	Proto-oncogene mutations
• t (8;21):AML-I ETO	• FLT 3activating mutation
• t (15;17): PML- RARA	• RAS mutations
• Inv 16: CBFB-MYH 11	• c-KIT (CD 117) activating mutations
• 11q23:MLL PTD	• NPMI mutations
• Rare – t(6;11), t(6;9), inv 3	• BAALC and ERG over expression
Point mutations in transcription factors	Tumor suppressor gene mutations
• Core-binding factor mutations	• P53, retinoblastoma
• CEBPA mutations	
• Wilms tumor (WT-1) mutation	

AML-I ETO, acute myeloid leukemia–eight twenty–one gene; BAALC, brain and acute leukemia, cytoplasmic; CBFB-MYH, core-binding factor gene–smooth muscle myosin heavy chain; CD cluster designation; CEBPA, CCAAT/enhancer binding protein alpha; ERG, ETS-related gene; FLT3, Fms-like tyrosine kinase 3; MLL PTD, mixed lineage leukemia-partial tandem duplication; NPM, nucleophosmin; PML-RARA, promyelocyte leukemia- retinoic acid receptor. RAS retinoic acid syndrome.

Review of the current literature suggests that the balanced translocations (t(15;17), t(8;21), inv(16), and *MLL* rearrangements) and nucleotide variants in *DNMT3A* and *TET2* most commonly occur in the founding clone at diagnosis, and are neither gained nor lost at relapse. In contrast, +8, +22, -X, Y, and nucleotide variants in *FLT3, NRAS/KRAS, WT1* and *KIT* frequently occur in subclones that either emerge or are lost at relapse.[8]

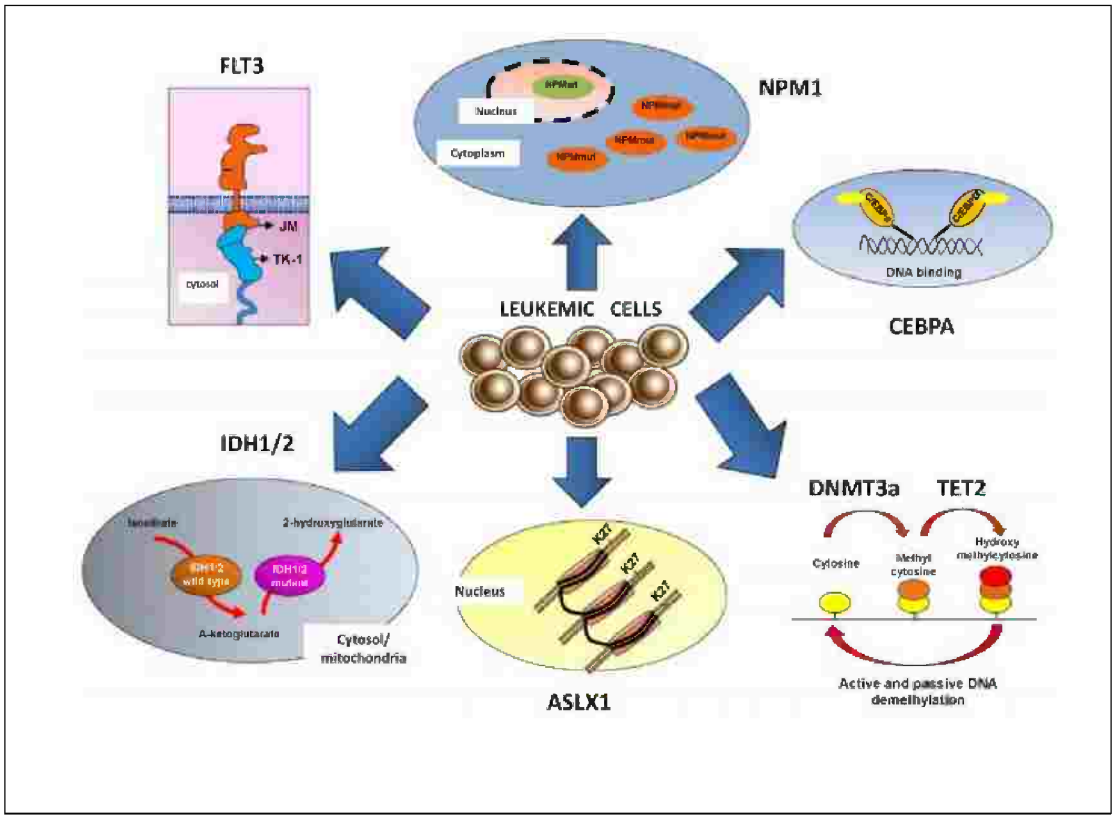


Figure 1: Schematic view of the most frequent mutated genes and their biological significance in AML cells [9]

Mutations that block differentiation:

Balanced translocations and inversions:

Core-binding factor acute myeloid leukemia:

Core-binding factor is a heterodimeric transcription factor complex that consists of 3 distinct DNA-binding CBFα subunits (RUNX1, RUNX2, and RUNX3),

and a common CBFβ subunit, which is non-DNA binding. RUNX1 has been known by a number of names including AML1, and CBFA2. The Human Genome Organization renamed it as runt-related transcription factor 1 (RUNX1). The binding affinity of RUNX1 subunit to the DNA promoter sequences is significantly increased by association with CBFβ, which does not directly interact with DNA and protects RUNX1 subunit from proteolysis. Core-binding factors are involved in hematopoietic development: animal studies have demonstrated that expression of the RUNX1 and CBFB genes is essential for the differentiation associated with normal hematopoiesis. The homozygous loss of either RUNX1 or CBFB alleles, in knockout murine models, resulted in failure to develop definitive hematopoiesis and in embryonic death. In the embryo, RUNX1 and CBFβ are required for the differentiation of definitive hematopoietic progenitors and hematopoietic stem cells from a "hemogenic endothelium.[10]

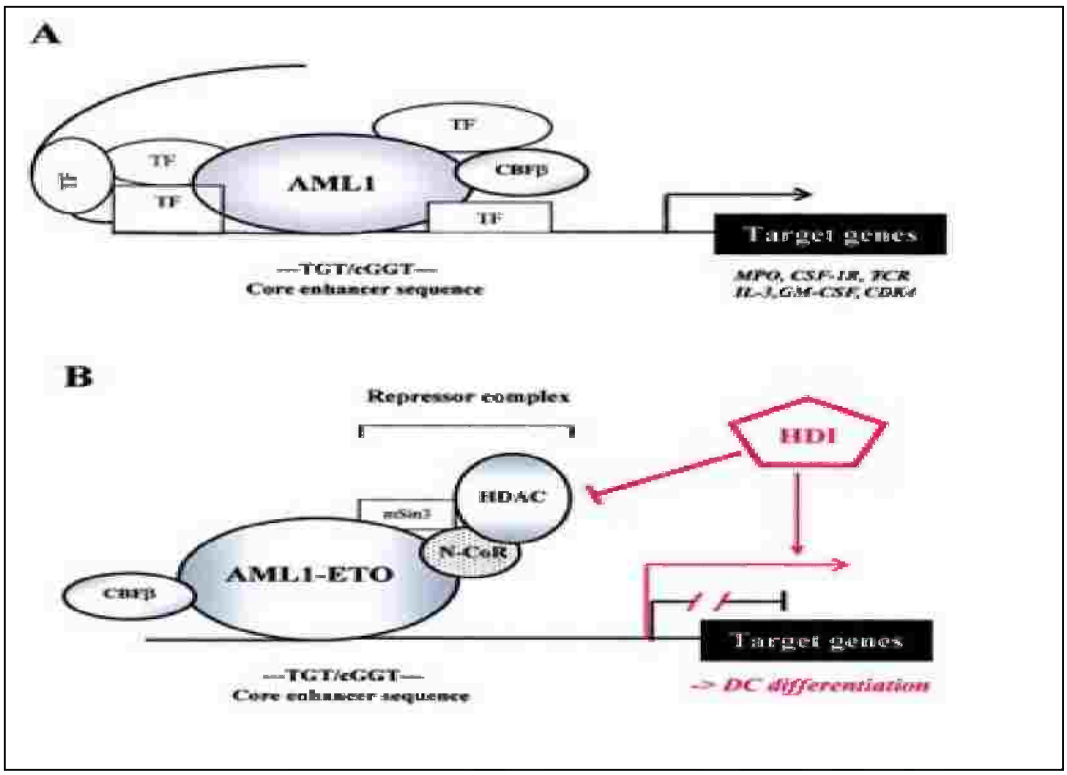


Figure 2: Schematic description of AML1 and AML1-ETO function

Schematic description of AML1 and AML1-ETO function. (A) In normal cell differentiation, AML1/core-binding factor (CBF)β functions as an enhancer-organizing protein that interacts with

several lineage-specific transcription factors [11] to induce gene transcription. Binding of the AML1/CBFβ complex to DNA usually leads to transcriptional activation of target genes whose transcription is regulated by AML1. (B) The AML1-ETO fusion protein retains the ability to bind the core-enhancer sequence and to heterodimerize with CBFβ. In contrast to wild-type AML1, the ETO component of AML1-ETO binds a corepressor complex that includes mSin3, N-CoR, and Hdi. This latter interaction results in suppression of genes whose transcription is normally activated by AML1-CBFβ, leading to a block of normal hematopoiesis. Application of histone deacetylase inhibitors (Hdi) (HDI in red) reverses transcriptional suppression, thereby supporting dendritic cell (DC) differentiation, while the leukemic fusion transcript is retained. Like AML1-ETO, TEL-AML1 also recruits N-CoR and histone deacetylase (HDAC), leading to a block in hematopoietic differentiation. [12]

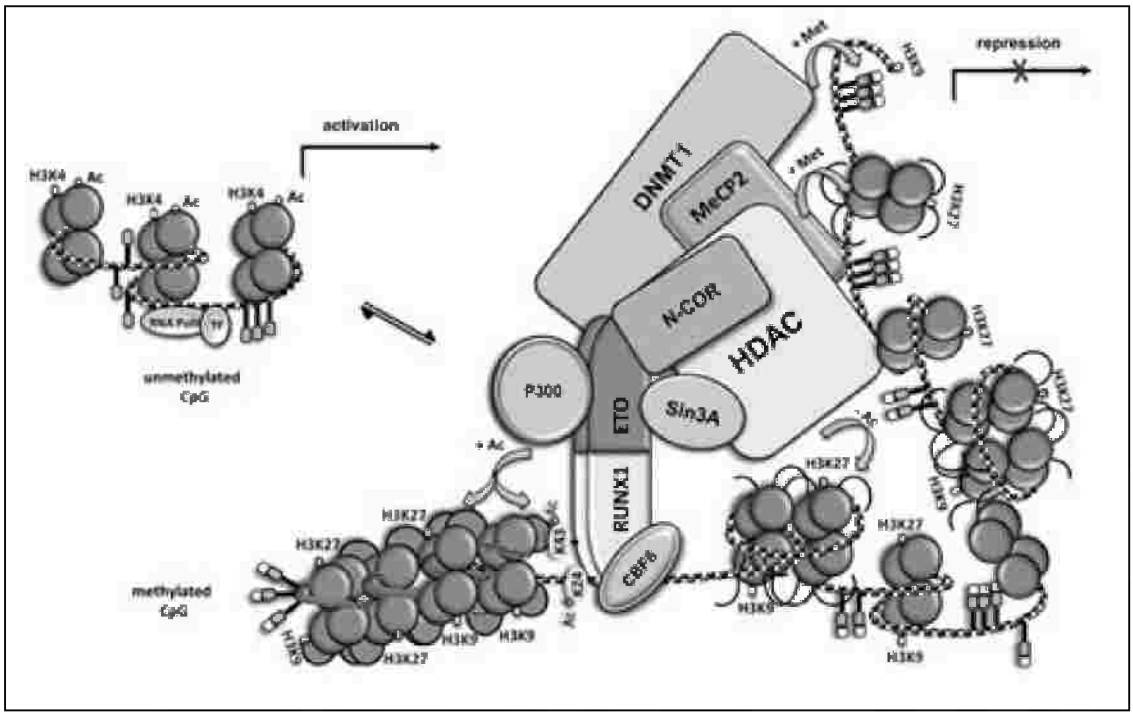


Figure 3:RUNX1/ETO assembles multiple epigenetic regulators [13]

RUNX1/ETO assembles multiple epigenetic regulators. Open chromatin (left side) and chromatin modification following RUNX1/ETO DNA-binding and high molecular weight complex formation. RUNX1/ETO recruits several proteins, including N-CoR, mSIN3A, HDAC, MeCP2 and DNMT1, into a high molecular weight complex that triggers chromatin condensation (right side), thereby repressing gene expression. Locus-dependent, RUNX1/ETO recruits the co-activator p300 and mediates acetylation of RUNX1 at the two conserved lysine residues and nearby histones, thereby promoting gene transcription. Ac, acetylation; Met, methylation; CpG, CpG site;TF, basal transcription factor.

Core-binding factor acute myeloid leukemia (AML) is cytogenetically defined by the presence of t(8;21) (q22;q22) or inv(16) (p13q22) / t(16;16) (p13;q22), commonly abbreviated as t(8;21) and inv(16), respectively. Upon detection of these clonal genetic abnormalities, the diagnosis of AML can be made regardless of the proportion of BM blasts.[14] In both subtypes, the cytogenetic rearrangements disrupt genes that encode subunits of core-binding factor.[10] The rearrangements t(8;21) and inv(16) involve the RUNX1/RUNX1T1 (AML1-ETO) and CBFB/MYH11 genes, respectively. These 2 subtypes are categorized as AML with recurrent genetic abnormalities. [10]

Among adults with de novo AML, t(8;21) and inv(16) are found in 7% and in 5% to 8% of the patients, respectively. The frequency of t(8;21) and inv(16) decreases in older patients, and only~7% of AML patients above the age of 60 years harbor one of both chromosome aberrations.[15]

These 2 cytogenetic subtypes are grouped together because of these common molecular features. However, the morphologic features of both are distinctly different. Patients with t(8;21) frequently present with the French-American-British (FAB) morphologic subtype M2 or acute myeloid leukemia with differentiation, while patients with inv(16) more often are diagnosed with FAB subtype M4Eo or acute myelomonocytic leukemia with eosinophilia.[10]

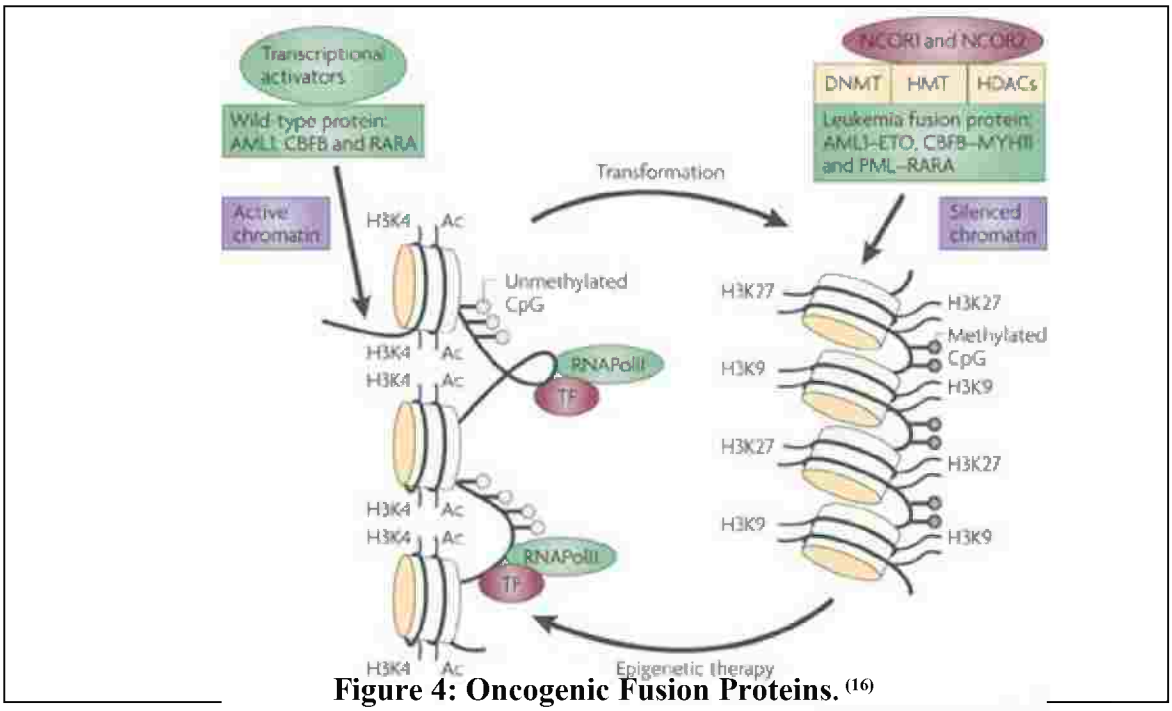


Figure 4: Oncogenic Fusion Proteins. [16]

Oncogeneic fusion proteins such as AML1–ETO, CBFB–MYH11 and PML–RARA recruit transcriptional co-repressor complexes (including nuclear receptor co-repressor 1 (NCOR1) and NCOR2) that result in the loss of histone acetylation and the acquisition of repressive histone modification marks, such as histone H3 lysine 9 (H3K9) methylation and H3K27 trimethylation, as well as DNA methylation, and thereby a closed chromatin structure. This leads to the transcriptional silencing of various target genes, including genes that are crucial for hematopoietic differentiation. Epigenetic or transcriptional therapy (targeting the fusion proteins, components of the co-repressor complexes and downstream effectors such as microRNAs) has the potential to reverse these changes, leading to histone acetylation, acquisition of active marks such as H3K4 methylation, an open chromatin structure with subsequent transcriptional activation and differentiation of the leukemic clone. Ac, histone acetylation; AML1, acute myeloid leukemia 1; CBFB, core binding factor-β; CpG, cytosine residues that precede guanosine; DNMT, DNA methyltransferase; HDAC, histone deacetylase; HMT, histone methyltransferase; PML, promyelocytic leukemia; RARA, retinoic acid receptor-α; RNApolII, RNA polymerase II; TF, transcription factor.

In general, CBF AMLs are considered to have a favorable prognosis when compared with other AML subtypes or with cytogenetically normal AML. In both subtypes, after standard induction therapy with cytarabine and anthracyclines, complete remission can be achieved in approximately 90% of the patient population. The CALGB study found that treatment with high-dose cytarabine has a

better outcome than treatment with intermediate- or low-dose cytarabine. In addition to the drug dose, the number of chemotherapy courses also plays a significant role in the outcome. Patients who receive 3 or 4 courses of high-dose cytarabine have a better outcome than those receiving only a single course. Marcucci et al[6] reported that 50% of patients with CBF AML have long-term survival when repetitive cycles of high-dose cytarabine are used as post-remission therapy. For both subtypes of CBF AML, patients have a high cytarabine in the consolidation phase.[10]

Secondary genetic changes in CBF-AML, mutations in the c-KIT [17] and FLT3 (FMS-like tyrosine kinase 3), have been associated with inferior outcome. While activating KIT mutations are found in approximately one-third of cases, the KIT receptor is expressed at significantly higher levels in CBF-AML compared with other subgroups. [18]

Risk-adapted stratification is used that is based on the genetic makeup of individual cases. Loss of sex chromosome, del(9q) and complex abnormalities were more common among patients with t(8;21), while +22 and +21 were more common with inv(16). [19] Although partial deletion of chromosome 9, del(9q), was considered to be an unfavorable prognostic factor, recent multicenter studies have failed to show any prognostic impact of del(9q) on complete remission and relapse-free survival. Patients with inv (16) and 1 or more secondary chromosome abnormalities, especially trisomy 22, had a lower risk of relapse than those with inv (16) solely. Patients with CBF AML and mutations in the KIT gene (exon 17) have a higher risk of relapse. [19]

Acute promyelocytic leukemia:

Acute promyelocytic leukemia is a subtype of acute myeloid leukemia (AML) characterized by distinctive morphology of blast cells, a life-threatening coagulopathy, and a specific balanced reciprocal translocation t(15;17), which fuses the *PML* (promyelocyte) gene on chromosome 15 to the *RARα* (retinoic acid receptor-α) gene on chromosome 17. Since the introduction of the differentiating agent all-*trans* retinoic acid (ATRA), the disease has become the most curable subtype of adult

AML. Despite this dramatic improvement in outcome, early death primarily because of hemorrhage before and during induction therapy remains the main cause for treatment failure.[20]

Acute myeloid leukemia with MLL-fusions:

Rearrangements of the mixed lineage leukemia (MLL) gene are found in approximately 10% of adult AML, especially in secondary acute leukemias that occur following treatment with topoisomerase II inhibitors. Except for the translocation t(9;11)[21], leading to a MLLT3-MLL fusion (also known as MLL-AF9), which is a unique WHO classification entity, and the translocation t(11;19)(q23;p13), the presence of an MLL rearrangement generally confers a poorer prognosis, although the analysis by MRC did not distinguish between the two different types of t(11;19), i.e. AML with t(11;19)(q23;p13.3) (MLL-MLLT1) and AML with t(11;19)(q23;p13.1) (MLLELL). While there are more than 60 known fusion partners of MLL, MLL-rearranged leukemias display remarkable genomic stability, with very few gains or losses of chromosomal regions. Therefore, recent studies suggest that MLL-rearranged leukemias are largely driven by epigenetic deregulation as several epigenetic regulators that modify DNA or histones have been implicated in MLL-fusion driven leukemogenesis.[18]

Point mutations in transcription factors:

Acute myeloid leukemia with CEBPA mutations

CCAAT/enhancer binding protein alpha (CEBPA) mutations are primarily found in CN-AML (10%-18%) and can be divided into two subgroups: single mutation, CEBPA (one-third of cases), and double mutation cases, CEBPA (two-thirds of cases). Typically, in CEBPA AML both alleles are mutated, one showing a N-terminal and one a C-terminal mutation. Recent studies showed that only $CEBPA_{dm}$ AML is an independent prognostic factor for favorable outcome. Therefore, the pattern of concurrent gene mutations is different in $CEBPA_{sm}$ (significantly higher frequency of NPM1 mutations and FLT3-ITDs than in $CEBPA_{dm}$) and global gene

expression studies revealed only a distinct prognostic feature for $CEBPA_{dm}$ AML cases. Consequently, only AML with $CEBPA_{dm}$ should be considered as a distinct entity and prognostic category that can be associated with additional genomic alterations such as GATA2 mutations. $CEBPA_{dm}$ patients may also not benefit from allogeneic HSCT based on the assumption that, in general, this approach may not improve outcome in favorable-risk AML. [22]

Mutations that promote proliferation:

Proto-oncogene mutations:

Molecular diagnostics are especially important in the large group of AML with normal karyotype which is composed of approximately 45% of all adult AMLs and had a very heterogeneous prognosis. [23]

To date, only diagnosis of *NPM1*, *CEBPA*, and *FLT3* mutations [23] has entered clinical practice and affects diagnosis. On the basis of characteristic clinical, pathologic, and biologic features, AML with *NPM1* mutation and AML with *CEBPA* mutation have been incorporated as provisional entities in the 2008 WHO classification of AML. Although *FLT3* mutations are not considered to define a distinct entity, they provide important prognostic information.[6]

Therefore, *NPM1*, *CEBPA*, c-KIT and *FLT3* mutations are recommended to be analyzed in clinical trials and in routine practice at least in patients with cytogenetically normal CN-AML who will receive treatment other than low-dose chemotherapy or best supportive care.[6]

Acute myeloid leukemia with NPM1 mutations:

Nucleophosmin 1 (NPM1) mutations are the most frequent mutations found in 25%-35% of adult AML, especially in CN - AML (45%-64%).[18] NPM1 mutations lead to abnormal cytoplasmic localization of the protein that is caused by disruption

of the N-terminal nucleolar localization signal of nucleophosmin and by the generation of a new nuclear export signal. [24]

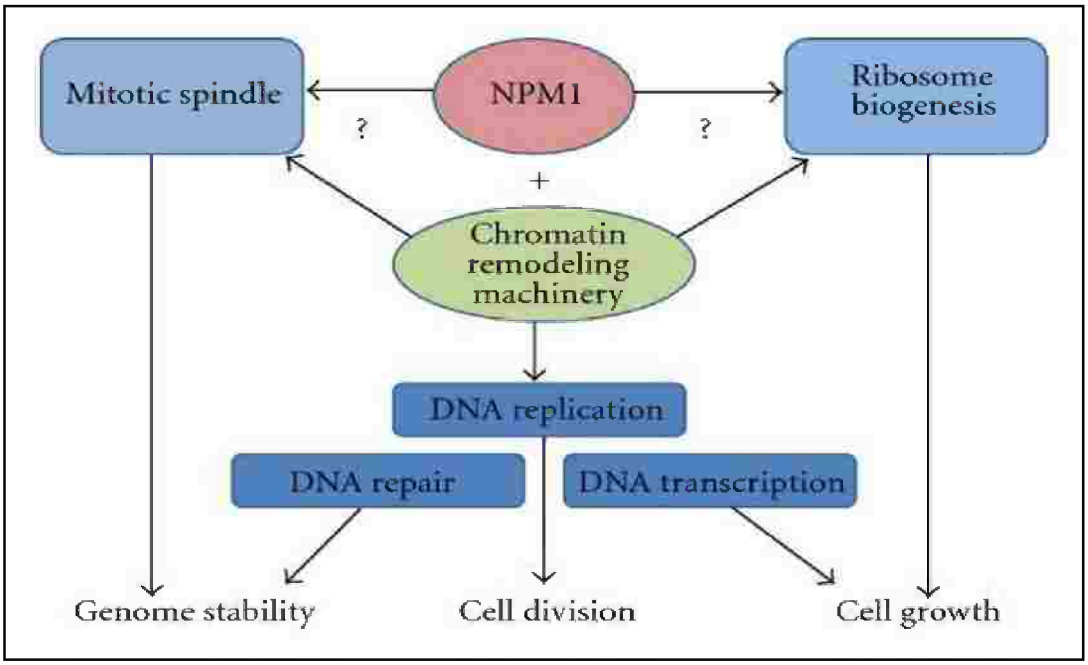


Figure 5: NPM1 is a multifunctional histone binding protein.

NPM1 can affect DNA replication, repair and transcription by interacting with the components of chromatin such as histones and chromatin remodeling proteins. NPM1 is also required for a controlled progression through mitosis and NPM1 can promote ribosome biogenesis. These effects may arise through the ability of NPM1 to bind histones at the centromeres or in the nucleolus, respectively. [25]

Patients with NPM1 mutation usually present with higher bone marrow blast percentages,[26] lactate dehydrogenase serum levels, and white blood cell counts, and blast cells typically show high CD33-antigen, but low or absent CD34-antigen expression. As NPM1 mutations without concurrent FLT3-ITDs have been shown to confer a superior outcome, the genotype "mutated NPM1 without FLT3-ITD[27] has been incorporated into the genetic favorable-risk category of the current AML recommendations. However, the prognostic value of the NPM1mut/FLT3-ITDneg genotype has to be revisited in the context of recently identified concomitant mutations, such as IDH and DNMT3A mutations. [4, 18]

shown that approximately 1/3 of ITDs insert in the TK1 rather than in the JM domain. The activation loop in the carboxyterminal lobe of the TKD is affected by point mutations, small insertions, or deletions mainly involving codon 835 and 836 in 11%-14% of CN-AML.[24]

When treated with conventional chemotherapy, prognosis of AML with FLT3-ITDs is significantly worse compared with AML without the mutation. Several studies have shown that the allelic ratio affects outcome in that AML with a high mutant–to–wild-type ratio do significantly worse compared with AML with lower ratios. Furthermore, one study showed that non-JM FLT3-ITDs are associated with a particularly poor outcome. The prognostic relevance of the FLT3-TKD mutation has remained controversial. [24]

Acute myeloid leukemia with c-KIT mutations:

The mechanism underlying KIT gene mutations that adversely affects the prognosis involves phosphorylation of the KIT receptor after physiologic binding of KIT ligand, which activates downstream pathways supporting cell proliferation and survival. KIT mutations represent not only a prognostic indicator but also a potential therapeutic target for the tyrosine kinase inhibitors.[10]

Approximately 20–30% of patients with t(8;21) or inv(16) have a c-KIT mutation. The cumulative incidence of relapse at 5 years has been estimated as 30–35% for patients with either t(8;21) or inv(16) but without a c-KIT mutation vs. 70% for t(8;21) patients with a c-KIT mutation and 80% for inv(16) patients with a mutation in exon 17 of c-KIT [32]. However because c-KIT mutations do not appear to affect outcome in the more common subtypes of AML [for example, normal cytogenetics (CN - AML)] it is probably practical to test for these mutations only once the patient is known to have inv (16) or t(8; 21). [32]

Acute myeloid leukemia with RUNX1 mutations:

The gene encoding runt-related transcription factor 1 (RUNX1) is targeted by chromosomal rearrangements such as t(8;21)(q22;q22) and intragenic mutations in

AML. The few studies evaluating the clinical impact of intragenic RUNX1 mutations suggest that these lesions may be associated with inferior outcome. The various frequencies of RUNX1 mutations reported in these studies may be due to patient selection and ethnic differences.[24]

Acute myeloid leukemia with DNMT3a mutations:

Mutations in DNA methyltransferase 3A [33] occur predominantly in patients with intermediate prognosis cytogenetics, including 25–35% of those with an NK [34]. Mutations of DNMT3A were identified by next-generation sequencing techniques. [24] DNMT3A, as well as DNMT3B and DNMT1, encode for methyltransferases catalyzing the addition of a methyl group to the cytosine residue of CpG dinucleotides. *DNMT3A* mutants showed reduced enzymatic activity resulting in decreased DNA methylation in several thousand genomic regions.[24] DNMT mutations are associated with mutations in NPM1 and ITDs of FLT3. They seem to convey poorer prognosis in NK AML, with this effect limited to patients who are not NPM1/FLT3 ITD negative [35].

Acute myeloid leukemia with IDH mutations:

Mutations in isocitrate dehydrogenase 1 (IDH1) or 2 (IDH2) also have been found chiefly in patients with intermediate prognosis cytogenetics, with two thirds of patients having a normal karyotype (NK). As with DNMT3a, the effect of mutations in IDH1 has been reported to be context dependent. Both IDH1 and IDH2 mutations result in a substrate shift to α- ketoglutarate (α-KG) with accumulation of 2-hydroxyglutarate (2-HG) that is a putative oncogenic metabolite. There is increasing evidence that the prognostic impact varies among the different mutations and also depends on the context of concurrent mutation. *IDH1* mutations confer inferior outcome in CN-AML, but the prognostic effect in the molecular subsets (with other molecular mutations) of CN-AML is controversial. [24] IDH1 mutations are independently associated with lower relapse rates in patients with, but higher relapse rates in patients without, FLT3 ITDs [36]. IDH2 illustrates the potential influence of

mutation site. While an IDH2 mutation at R140 has been found to confer on patients with an otherwise intermediate prognosis a probability of relapse similar to that found in patients with inv(16) or t(8;21), a mutation at R172 confers a probability of relapse resembling that seen in patients with unfavorable cytogenetics [32].

Acute myeloid leukemia with WT1 mutations:

The prognostic influence of some mutations, for example in the WT1 gene, has differed in different series. [37] More than a decade ago, King-Underwood et al reported *Wilms' Tumor 1* (*WT1*) gene mutations in 10% of acute leukemias. Recently, further studies were performed on larger cohorts showing mutations primarily in CN-AML with a frequency of 10%-13%. The prognostic impact of the mutation remains somewhat inconclusive. Whereas in the studies by MRC, CALGB, and Acute Leukemia French Association (ALFA) groups, a negative impact of the mutation on OS was shown, no impact was found in a study of 617 CN-AML by AMLSG. The latter study only found a possible negative impact in AML with concurrent *WT1* and *FLT3*-ITD mutations. [24]

Acute myeloid leukemia with TP53 mutations

Recently, *tumor protein p53* (*TP53*) mutation and/or loss of the *TP53* allele was detected in 69%-78% of AML cases with a complex karyotype (CK-AML), whereas *TP53* mutations were very rare in non-complex karyotype AML (2.1%). Based on an integrative *TP53* mutational screening analysis and array-based genomic profiling in 234 CK-AMLs, 70% of cases had altered *TP53*; 60% *TP53* mutations and 40% *TP53* losses of CK-AMLs. [38] As *TP53* - altered CK-AMLs are characterized by a higher degree of genomic complexity, they more frequently exhibit a monosomal karyotype (MK), which previously was associated with poor AML outcome. TP53 alterations are also associated with older age, specific DNA copy number alterations, and dismal outcome. In multivariable analysis, TP53 alteration is the most important prognostic factor in CK-AML, outweighing all other variables, including the MK

category. This very unfavorable prognosis of TP53 mutation was recently confirmed in an independent study showing an overall survival (OS) at three years of 0%. [18]

PROGNOSIS IN ACUTE MYELOID LEUKEMIA

An increasing number of genomic aberrations and gene mutations have been identified that cause epigenetic changes and lead to deregulated gene expression. For example, small genomic losses pointed to a relevant role of the *TET2* gene[39] and next generation sequencing (NGS) helped to identify *IDH1* and *DNMT3A* mutations in AML. [40, 41]

These recent insights further highlighted the molecular heterogeneity of AML and showed that individual patients present with a distinct and almost unique combination of somatically acquired genetic aberrations. While some of these are known to perturb a variety of cellular processes of the hematopoietic progenitor cells, including mechanisms of self-renewal, proliferation, differentiation, epigenetic regulation, DNA repair, and RNA splicing, others most likely represent passenger mutations that do not significantly contribute to the disease. In recent years, this growing genetic information has started to translate into the clinic. The current World Health Organization (WHO) classification categorizes more than half of AML cases on the basis of the underlying genetic defects, which in part define distinct entities of clinical importance. First, cytogenetic and molecular genetic changes represent powerful prognostic markers, and second some genetic and epigenetic aberrations can be targeted by novel therapeutic approaches, such as tyrosine kinase inhibitors (TKIs) and demethylating agents. [42]

However, there are still limitations regarding the use of genomic biomarkers in clinical practice as for many novel markers the prognostic impact so far has only been evaluated in retrospective studies. In addition, several new markers still need to be interpreted cautiously as first studies did not take into account interactions with other molecular markers, and often the analysis was based on relatively low patient numbers that might show a selection bias. Furthermore, for improved clinical decision-making there is an unmet need for predictive markers that can be attributed to the clinical benefit of a specific treatment. This might contribute significantly to an improvement in the treatment of AML that has been slow over the past decade with a few subgroups as exceptions, such as younger patients with more favorable genetic disease.[9] Not only novel targeted therapies, but also dose escalation of daunorubicin,

the use of alternative nucleoside analogs, antibody-directed chemotherapy as well as allogeneic transplant concepts will benefit from a biomarker guided personalized treatment approach. Finally, a more detailed molecular characterization will provide also the opportunity to more precisely monitor minimal residual disease (MRD) in all AML patients.[9]

Risk status based on cytogenetic and molecular abnormalities

Table 2: Risk status based on cytogenetic and molecular abnormalities[43]	
Risk status	Cytogenetic and molecular abnormalities
Better risk (favorable)	Inv (16), or t(16;16)
	t(8; 21)
	t(15; 17)
	Normal karyotype with NPM1 mutation or isolation CEBPA mutation in the absence of FLT3 –ITD
Intermediate risk	Normal cytogenetics
	+8
	-Y
	t(9; 11)
	t(8; 21), inv (16), t(16; 16) with c-KIT mutation
	Not defined as better or poor risk
Poor risk	Complex karyotype (≥ 3 clonal chromosomal abnormalities)
	Monosomal karyotype (at least 3 monosomies or one monosomy +1 structural abnormality)
	-5, 5q-, -7, 7q-
	11q 23- non t(9; 11)
	Inv (3), t(3; 3)
	t (6; 9)
	t (9; 22)
	Normal cytogenetics with FLT3- ITD mutations
CEBPA, CCAAT/ enhancer binding protein alpha; c- KIT, CD117 receptor tyrosine kinase; FLT3. Fms tyrosine kinase 3- internal tandem duplications; NPM1, nucleophosmin.	

Impact of gene mutations in acute myeloid leukemia with normal karyotype [4]

Table 3: Frequency and prognostic impact of gene mutations in acute myeloid leukemia with normal karyotype

Gene mutation	Frequency (%)	Prognosis
NPM1	45-62	Favuorable in FLT3-ITD- Adverse in FLT3-ITD+
FLT3 –ITD	20-30	Adverse
FLT3-TKD	11-14	Unclear
DNMT3a	25-35	Adverse
IDH1/2	8-15	Varied based on combination with other genotypes
TET-2	12-27	Varied based on combination with other genotypes
MLL1	5-11	Adverse
CEBPA	15-20	Favourable
NRAS	11-25	Unclear
WT1	≈10%	Unclear, likely adverse
RUNX	14-34	Unclear

CEBPA, CCAAT/ enhancer binding protein alpha; FLT3, Fms tyrosine kinase 3-internal tandem duplications; NPM1, nucleophosmin; FLT –TKD, Fms tyrosine kinase 3 tyrosine kinase domain; IDH1/2, isocitrate dehydrogenase 1 and 2; MLL1, mixed lineage leukemia -1; NPM1, nucleophosmin; NRAS, neuroblastoma rat sarcoma; RUNX, runt – related transcription factor;TET-2, ten eleven translocation 2; WT1, Wilms tumor 1.

TREATMENT

Treatment for AML generally is divided into remission induction and post remission therapy. Standard remission induction regimens in the United States for all AML subtypes, excluding APL, almost always include 7 days of infusional cytarabine and 3 days of an anthracycline, commonly known as the "7+3" strategy. This strategy results in complete remission (CR) in 70%-80% of adults <60 years of age and 30%-50% of selected adults >60 years of age with a good performance status. The Cancer and Leukemia Group B (CALGB) established that 3 days of daunorubicin and 7 days of cytarabine were more effective than 2 and 5 days, respectively, and that 10 days of cytarabine was not better than 7 days. Also, 100 mg/m^2 of cytarabine for 7 days was as effective as 200 mg/m^2 for the same duration. Daunorubicin at a dose of 30 mg/m^2 was inferior to 45 mg/m^2, and recently, daunorubicin 90 mg/m^2 has been shown, in large cooperative group trials, to be superior to 45 mg/m^2 even in selected patients >60 years of age. Many modifications to the standard "7+3" backbone have been attempted. Remission rates were similar or slightly improved when idarubicin or mitoxantrone was substituted for daunorubicin, but there was no convincing improvement in overall survival when equivalent doses were used. Randomized prospective trials also failed to demonstrate that induction with high-dose cytarabine (HiDAC) improved survival in most patient subgroups compared with standard induction. Similarly, addition of 6-thioguanine, etoposide, to the anthracycline or cytarabine backbone did not improve overall survival. Finally, despite compelling scientific rationale, neither the addition of multidrug resistance modulators nor the addition of cytokines to chemotherapy (granulocyte-colony stimulating factor priming) has improved outcomes in AML to date. Trials combining targeted antibodies and *FLT3* inhibitors with chemotherapy are ongoing. Results for the targeted agent gemtuzumab ozogamicins have been conflicting to date but may benefit specific subgroups of patients. [(1)]

Once remission has been achieved, further therapy is required to prevent relapse. Options include repeated courses of consolidation chemotherapy or hematopoietic stem cell transplantation (HSCT). Autologous HSCT permits escalation to myeloablative doses of chemotherapy, and allogeneic HSCT allows combination of myeloablative chemotherapy with a graft-versus-leukemia effect from

the donor cells. Several studies have prospectively evaluated the role of intensive consolidation with HiDAC. The CALGB randomized patients in first remission to four courses of cytarabine using either a continuous infusion of 100 mg/m2 for 5 days or a 3-hour infusion of 400 mg/m2 or 3 g/m2 twice daily on days 1, 3, and 5. Significant CNS toxicity was observed in patients >60 years old randomized to the high dose arm, and thus, this regimen is not recommended for older patients. In patients <60 years old, there was a significant improvement in disease-free survival associated with the high-dose regimen, and this was most pronounced in patients with favorable cytogenetics, including t(8;21) and inv(16). [1] Although it has become standard to offer at least two cycles of HiDAC at 1 to 3 g/m2 to younger patients with AML, there are no clear data defining the optimal number or intensity of HiDAC cycles. Randomized trials from the United Kingdom Medical Research Council failed to demonstrate that three cycles of HiDAC consolidation were better than two cycles. Although it is clear that patients with CBF leukemias specifically benefit from HiDAC, some of these patients also have mutations in *KIT*, which are associated with an inferior outcome; clinical trials of chemotherapy combined with tyrosine kinase inhibitors are ongoing in these patients. Consolidation chemotherapy, in general, has not been proven to be of benefit for patients >60 years old, but older patients able to tolerate additional treatment often are offered one or two cycles of 5 days of cytarabine combined with 2 days of an anthracycline after induction. Maintenance therapy outside of APL has not been adopted. Two pediatric randomized trials from the Leucémies Aiguës Myéloblastiques de l'Enfant (LAME) and the Children's Cancer Group (CCG) failed to demonstrate that maintenance therapy improves outcomes. [1]

Several studies of postremission therapy in AML have compared intensive chemotherapy consolidation to HSCT by assigning younger patients with a human leukocyte antigen (HLA)-matched sibling donor to allogeneic HSCT and randomizing other patients to chemotherapy or autologous HSCT. Meta-analyses have shown that autologous HSCT decreases relapse risk but increases treatment-related mortality compared with chemotherapy consolidation, thus resulting in similar overall survival rates of approximately 40%-45% at 3-5 years. There is no specific indication for its

use in any prognostic subgroup. [1] Allogeneic HSCT is probably the most effective antileukemic therapy currently available and offers a combination of the therapeutic efficacy of the conditioning regimen and the graft-versus-leukemia effect from the donor cells. It is, however, associated with significant morbidity and mortality. A comprehensive meta-analysis of prospective clinical trials of allogeneic HSCT in AML patients in first CR, evaluated 24 trials and more than 6,000 patients. In this analysis, allogeneic HSCT resulted in significantly improved 5-year overall survival, from 45% to 52% for patients with intermediate-risk cytogenetics and from 20% to 31% in patients with poor-risk cytogenetics. There was no benefit of allogeneic HSCT for patients with good risk cytogenetics. Retrospective analyses of uniformly treated patients have shown that allogeneic HSCT was also beneficial for cytogenetically normal AML patients with *FLT3*- ITD+, *FLT3*-ITD–/*NPM1*–, and *FLT3*-ITD–/*CEBPA*–, and prospective trials using molecular and cytogenetic risk stratification are under way. Other efforts are focusing on the use of alternative donor sources of stem cells to allow allogeneic transplant options for patients without fully matched sibling or unrelated donors. Trials utilizing partially matched– related donors, including haploidentical donors, as well as cord blood as sources of stem cells are under way by national cooperative transplant groups. Finally, using nonmyeloablative or reduced-intensity conditioning regimens is another way to broaden the application of allogeneic SCT toward patients who may not be medically fit to undergo a full preparative regimen.[1]

Acute myeloid leukemia in older patients: conventional and new therapies

Choice of treatment

Despite the reluctance to treat older patients with intensive chemotherapy because of toxicity concerns, induction of a complete remission (CR), even if short-lived, is an appropriate goal for most AML patients over 60 years of age. This concept was established in the late 1980s based on the results of the EORTC AML-7 trial which prospectively compared induction therapy with daunorubicin, and cytarabine *versus* supportive care with palliative chemotherapy (hydroxyurea or low-dose cytarabine) in patients over 65 years of age.[13] The patients who received induction chemotherapy had a higher CR rate (58% *vs.* 0%), lower incidence of early mortality (3 of 31 *vs.* 18 of 29), longer median survival (21 *vs.* 11 weeks) and greater chance of survival at 2.5 years (13% *vs.* 0%). Importantly, there was no difference in the number of days that patients were hospitalized. Furthermore, registry data from nearly 3000 unselected older patients in Sweden showed reduced rates of early mortality for those who received intensive chemotherapy *versus* palliative care, as well as improved long-term survival in geographical regions where the use of intensive treatment approaches was more common. [44] Thus, achieving CR is a requisite end point for better survival and improved quality of life in elderly AML, and data from large population-based studies have validated the use of intensive chemotherapy over less intensive treatment approaches in patients up to the age of 80 years. [44]

Although it is clear that intensive chemotherapy produces the highest response and survival rates in selected elderly patients with AML, it is ineffective and highly toxic in many others. The challenge is to appropriately identify which patients, based on their disease biology and clinical characteristics are likely to benefit more from intensive chemotherapy and which require alternative treatment approaches. Several risk scores are available that account for age, performance status, cytogenetics, secondary AML and other covariates to arrive at a prognosis for patients over 60 years of age treated with intensive chemotherapy. Despite the differences in variables and end points and methods used, these tools can be used to more accurately

individualize the treatment prospects. Patients with the expectation of a low early mortality, high CR rate, and a reasonable long-term survival should be treated with intensive chemotherapy, while those with the expectation of a high risk of early mortality or a poor chance of long-term survival should be offered low-intensity investigational therapy. [44]

Conventional remission induction therapy

For over 30 years, the "3+7" regimen combining daunorubicin (45-50 mg/m^2 for 3 days) and cytarabine (100-200 mg/m^2 by continuous infusion for 7 days) has been the mainstay of induction therapy for older patients with AML. [1] On average, this regimen offers older patients a CR rate of 40-65% with an attendant treatment-related mortality of 15-20%, a median survival of 8-12 months, and a less than 15% probability of sustained remission for three years. Multiple attempts have been made to improve outcome by substituting newer anthracyclines (idarubicin or mitoxantrone) for daunorubicin, escalating the dose of cytarabine, adding other cytotoxic drugs, and priming with growth factors, but none of these strategies has emerged as convincingly superior to "3+7". However, a recent combined analysis of two randomized ALFA trials (9801 and 9803), enrolling a total of 727 AML patients aged 50 years and over (median 67 years) showed a somewhat superior long-term outcome with idarubicin compared to daunorubicin (cure rate 16.6% *vs.* 9.8%; P=0.018). Interestingly, the long-term impact of idarubicin was also evident in the cohort of patients under 65 years of age, although all of the younger patients in the control arm received daunorubicin at higher doses (80 mg/m^2 x 3). [45]

Older patients with AML:

Most patients with AML are >60 years old, and their prognosis is dismal, with median survival times of only 8-12 months among the most "fit" patients. Older patients have a high frequency of poor prognostic features, including antecedent hematologic disorders, unfavourable cytogenetics, and multidrug resistance (*MDR1*) phenotypes. Also, older patients are often less able to tolerate intensive chemotherapy because of medical comorbidities, polypharmacy, poor performance status, and limited social supports. There is no universally accepted standard of care for the treatment of older patients, but they generally are offered either conventional "7+3" induction, hypomethylators, repeated cycles of low-dose subcutaneous cytarabine, supportive care with antibiotics and transfusions, hospice care, or an investigational trial. Although remission can be attained in ~50% of selected older patients with a good performance status using 7+3, relapse is almost certain, and <10% of patients are long-term survivors. Major cooperative group trials, which generally favour patients <75 years old with de novo AML and a good performance status, show 3- to 5-year overall survival rates of only 10%-20%. Many older patients are not offered any treatment for AML despite randomized data clearly demonstrating a survival benefit favoring treatment with chemotherapy over supportive care in this population. Clinical experience suggests that quality of life is better for those who achieve CR, but data are sparse. Although there are clearly frail and debilitated older patients who cannot tolerate any treatment, emerging data suggest that age alone should not be used as the major determinant of treatment because several intensive options, including intensified doses of daunorubicin and reduced-intensity stem cell transplantations, are both feasible and effective in selected patients >60 years old. Many, if not most, older patients with AML fail to benefit from therapy because of lack of therapeutic efficacy, not intolerable toxicity. Novel therapies are clearly needed for this population, and there are many ongoing clinical trials with cytotoxics, antibodies, farnesyltransferase inhibitors, hypomethylating agents, and nonmyeloablative transplantations. Older AML patients should be encouraged to participate in clinical trials whenever possible.[1]

PROGNOSTIC VALUE AND IMPACT ON TREATMENT DECISION OF SELECTED MOLECULAR MARKERS IN ADULT AML[24]

Table 4: Prognostic value and impact on treatment decision of selected molecular markers in adult AML		
Biomarker	**Prognostic significance**	**Impact on therapy**
CBF-AML: t(8;21)(q22;q22); RUNX1-RUNX1T1 and inv(16)(p13.1q22) or t(16;16)(p13,1q22); CBFB-MYH11	Favorable prognosis in younger and older patients In AML with inv(16), additional trisomy 22 predicts superior RFS	"3 + 7" induction followed by repetitive cycles of high-dose cytarabine considered as a widely accepted standard therapy for patients with CBF-AML.
	Secondary KIT and possibly also FLT3 mutations associated with inferior outcome in most but not all studies	In general, patients are not candidates for allogeneic HSCT; HSCT may be considered in individual patients with high-risk factors (eg, elevated WBC counts, molecular disease persistence) and low transplantation- related mortality
	High relapse probability in patients with molecular disease persistence	Older patients with CBF- AML benefit from intensive conventional chemotherapy Addition of anti-CD33 antibody GO in one trial (MRC15) significantly improved OS KIT inhibitor dasatinib in combination with intensive induction and consolidation therapy in phase II clinical trials

FLT3-ITD	Unfavorable prognosis	Allogeneic HSCT appears to improve outcome in younger adult patients (no data available for elderly patients)
	Particular poor outcome in AML with high burden of mutated FLT3-ITD allele (high mutant to wild-type allelic ratio as assessed by DNA fragment analysis)	Patients should be entered on clinical trials with FLT3 tyrosine kinase inhibitors whenever possible; first-generation (eg, midostaurin, lestaurtinib, sorafenib) and second-generation TKI (quizartinib) are currently being evaluated in phase II and III clinical trials
	AML with FLT3-ITD located outside the JM (non-JM ITD, approximately 30% of cases) appear to do significantly worse than those with AML with JM-ITD	
NPM1	Genotype "mutated NPM1-ITD" (in CN-AML) associated with favorable outcome without FLT3	Similar to CBF-AML, standard induction therapy followed by repetitive cycles of high-dose cytarabine is a reasonable first-line treatment option in patients -ITD" (CN-AML) without FLT3 with the genotype "mutated NPM1
	Impact of concurrent gene mutations, eg, in the IDH1, IDH2, DNMT3A, and TET-2 genes currently under investigation	Patients with such molecular favorable-risk CN-AML may not benefit from allogeneic HSCT in first CR; it may be considered in individual patients (eg, those with molecular disease persistence, low transplantation-related risk, clinical

		trial)
	NPM1 mutations in older patients associated with CR achievement and better outcome, even in patients above the age of 70 years	Older patients with NPM1-mutated AML benefit from intensive conventional chemotherapy
CEBPA	CEBPA (CN-AML) associated with favorable outcome Impact in older patients under investigation	Similar to NPM1-mutated AML, standard induction therapy followed by repetitive cycles of high-dose cytarabine is a reasonable first-line treatment option; patients may not benefit from allogeneic HSCT in first CR
IDH1, IDH2	Prognostic impact varies among the different IDH mutations. It appear to confer higher risk of relapse and inferior OS in CN-AML, however, the effect in the various molecular subsets of CN-AML is controversial	Not known IDH inhibitors in preclinical development
	IDH2 R172 mutations are only rarely found in concert with other known recurring gene mutations (ie, NPM1, CEBPA , FLT3 -ITD); they are	

	associated with inferior CR rate; impact on outcome unclear	
	Prognostic impact of IDH2 SNP rs11554137 (located in the same exon as the R132 mutation) in one study found to be associated with inferior outcome in molecular high-risk CN-AML (either NPM1 mutations controversial IDH1 wild-type or FLT3-ITD positive)	
WT1	Prognostic significance somewhat controversial; most studies report a negative prognostic impact	Not known
	Additional studies, preferentially large inter-individual patient meta-analyses, needed to explore the prognostic impact by different post-remission therapies	

	WT1 SNP rs16754 located in the mutational hot spot of WT1 in exon 7 in one study found to be associated with favorable prognosis in patients with CN-AML	
RUNX1	Prognostic impact under investigation Three studies showed an association of RUNX1 mutations with lower CR rate and adverse outcome	Not known One study (AMLSG) suggested that allogeneic HSCT may improve outcome; finding needs to be confirmed
TET-2	One study (CALGB) found a negative impact in the subset of molecular favorable-risk (mutated NPM1without FLT3 -ITD) AML where patients relapsed earlier and had a shorter overall surivial; another study (AMLSG) found no impact where neither patients remission nor survival was affected byTET-2 mutations	Not known
DNMT3A	DNMT3A mutations associated with intermediate-risk cytogenetics (in normal	Not known

	karyotype), and with FLT3, NPM1, and IDH mutations. Prognostic significance under investigation	
CEBPA, CCAAT/ enhancer binding protein alpha; FLT3, Fms tyrosine kinase 3- internal tandem duplications; NPM1, nucleophosmin; FLT –TKD, Fms tyrosine kinase 3 tyrosine kinase domain; IDH1/2, isocitrate dehydrogenase 1 and 2; MLL1, mixed lineage leukemia -1; NPM1, nucleophosmin; NRAS, neuroblastoma rat sarcoma; RUNX, runt – related transcription factor;TET-2, ten eleven translocation 2; WT1, Wilms tumor 1.		

HUMAN LEUCOCYTE ANTIGEN-G

Definition

The HLA-G is non-classical MHC class I protein that has been originally described as being selectively expressed on the invasive trophoblast at fetal-maternal interface at the beginning of pregnancy. [46] A few years later, HLA-G protein was detected after fertilization as early as oocyte stage, [47] and its presence was associated with efficient implantation of fertilized oocyte in uterine mucosa.[48] Its major contribution to successful pregnancy was also pointed out by both following observations:

1- Its reduced expression in pregnancy disorders such as preeclampsia and recurrent spontaneous abortion. [49]

2- HLA-G expression by trophoblast was shown to protect fetus from decidual NK cell attack. [50]

Since then, the expression of HLA-G has been extended to other tissues at immune privileged sites such as: thymus, [51] cornea, [52] pancreas, [53] and the erythroid and endothelial precursors. [54, 55]

Moreover, its ability to inhibit the effectors functions of decidual NK cells has been demonstrated for allogeneic NK, T, and antigen-presenting cells (APC), [56] which has set HLA-G as a molecule of immune tolerance.

In this regard, HLA-G protein was suggested to be a way used to evade the host immune reaction in pathological situations such as infectious diseases, [57]transplantation [58] and cancer. [59]

Tumors employ different strategies to prevent immune responses including tumor-induced impairment of antigen presentation, the activation of negative co-stimulatory signals and the elaboration of immunosuppressive factors.

Recently, Schreiber and colleagues [60] propose the cancer immunoediting hypothesis which integrates the different mechanisms of tumor immune escape with the cancer immunosurveillance theory. [61]

The cancer immunoediting concept consists of three phases: elimination, equilibrium and escape. The elimination phase corresponds to cancer immunosurveillance and implements cells from innate and adaptative immunity which recognize and destroy tumor cells. In case of partial eradication of tumor cells, equilibrium between the tumor and the immune system develops, that leads to the production of less immunogenic tumor cell clones.

Finally, these tumor cell variants escape the antitumor response, which results in tumor growth. The expression of the immunotolerant HLA-G protein at tumor site represents one of the immunosuppressive strategies mediated by tumors. [62]

Structural features of HLA-G

In addition to its restricted tissue distribution and its immunotolerant properties, HLA-G has structural particularities. The primary transcript of HLA-G gene is alternatively spliced producing seven mRNA encoding four membrane-bound protein isoforms: HLA-G1 to -G4 and three soluble ones: HLA-G5 to -G7. [63-65]

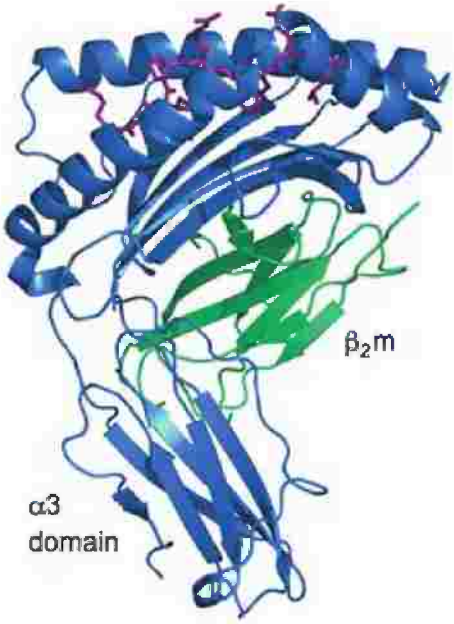


Figure 6: Overview of the structure of human leukocyte [66]

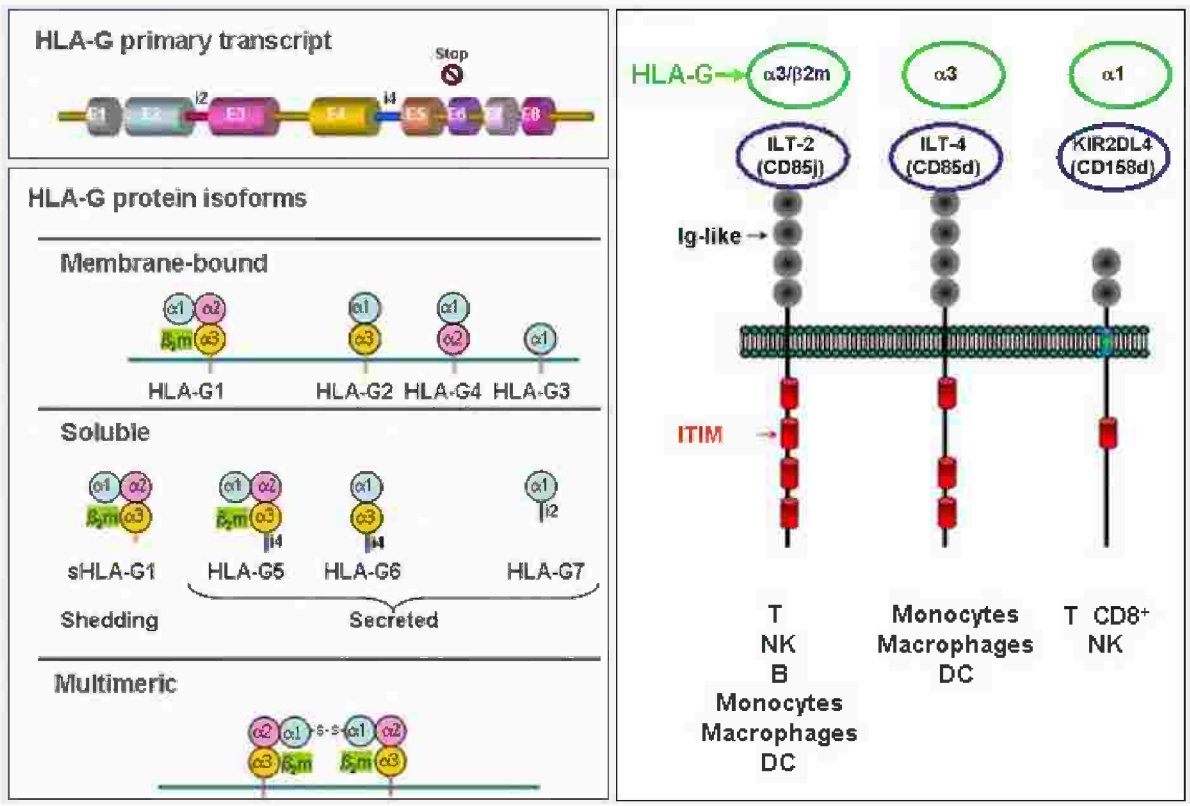


Figure 7: HLA-G Isoforms and their receptors

B= B lymphocytes, DC= Dendritic Cells, T= T Lymphocytes, NK= Natural Killer

At the structural level, HLA-G1 and its soluble counterpart HLA-G5 are similar to classical HLA class I protein as they include three extracellular domains, the third domain being non covalently associated to β2 microglobulin. Therefore, among the HLA-G protein isoforms, HLA-G1 and -G5 have been the most studied. Numerous monoclonal antibodies recognizing both isoforms in their properly folded conformation have been developed , [67] which allowed not only to analyze their pattern of tissue distribution but also to demonstrate the direct role of HLA-G in inhibiting immune responses, by blocking the interactions between HLA-G and its receptors. [68-70] To date, three receptors for HLA-G have been described: one member of the killing immunoglobulin-like receptor (KIR) family : KIR2DL4, which is expressed at NK and CD8+ cell-surface, [71] and two members of the immunoglobulin-like transcript (ILT) receptor family : ILT-4 (CD85d) present on myeloid cells , [72] and ILT-2, on lymphoid and myeloid cells [73] while KIR2DL4 is specific for HLA-G, [74] ILT-2 and ILT-4 also bind some HLA class I alleles but with a much lower affinity than HLA-G . [75]

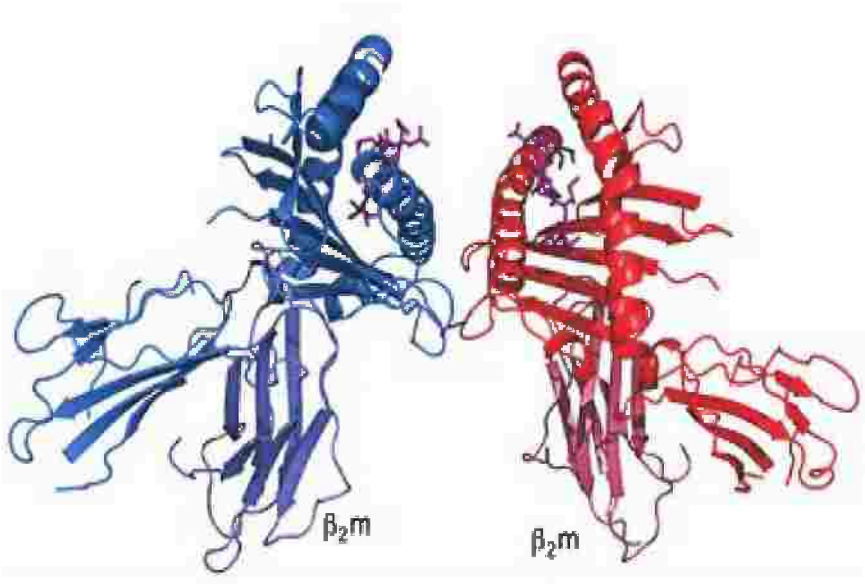


Figure 8: HLA-G dimer. The Cys42 disulfide-linked homodimer

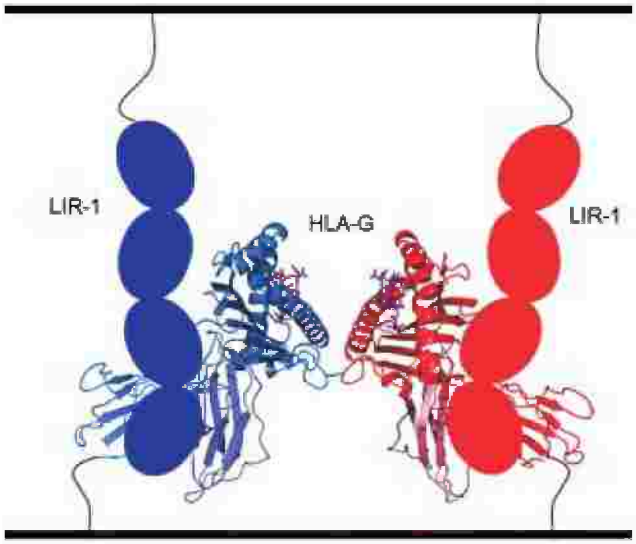


Figure 9: LIR-1 binding to HLA-G dimer. [66]

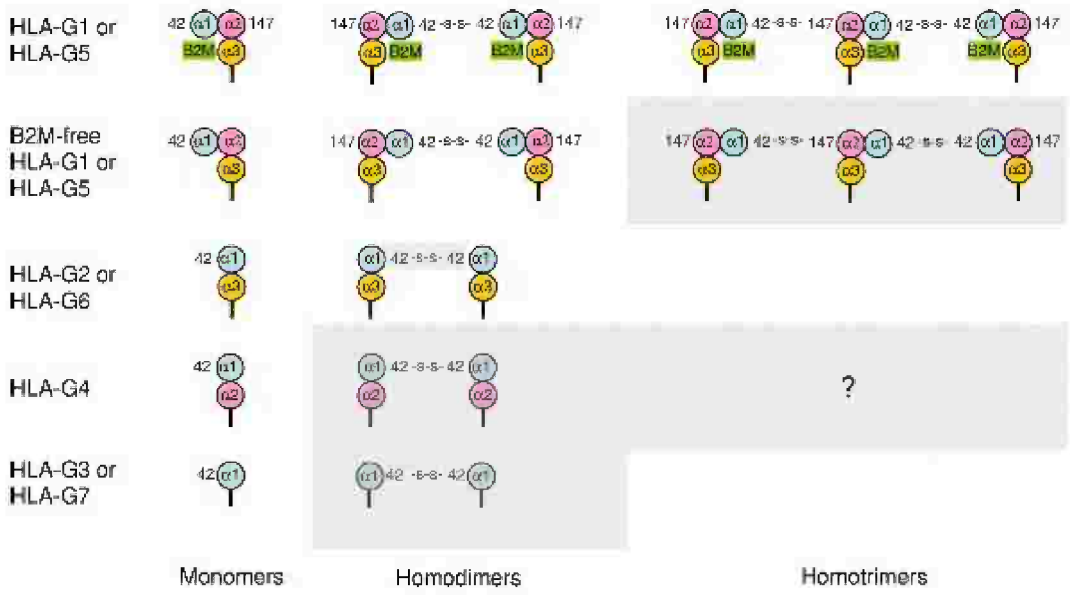


Figure 10: Homomultimeric structures of HLA-G. [76]

In the past few years, it has been highlighted that HLA-G1 forms dimers at cell-surface of transfected cells but also of trophoblast cells. [77, 78] The HLA-G dimers exhibit higher overall affinity to ILT-2 and -4 receptors than the monomers by significant avidity effects, [79-82] suggesting that the active conformation of HLA-G is

the dimeric form. In addition, the association of HLA-G heavy chain with β2 is required for interaction with ILT-2, but not for binding to ILT-4. [81] Recently, soluble HLA-G1 (shed HLA-G1) and HLA-G5 proteins were detected in body fluids such as plasma from hepato-renal transplanted patients [79] and malignant effusions, [83] through the development of enzyme-linked immunosorbent assays (ELISA) of which two HLA-G-specific ones were validated during an international workshop. The other HLA-G isoforms differs from HLA-G1 and -G5 by the lack of one (HLA-G2, -G4, and-G6) or two (HLA-G3 and -G7) extracellular domains. Their conformational structure remains to be determined. Although their detection is still difficult, the availability of an antibody directed against a peptide in the a1 domain common to all HLA-G isoforms allowed their characterization. [62, 84]

Cell-surface expression of these truncated isoforms is probably dependent on the type of cell in which they are expressed. Their detection as membrane bound proteins was related to the same ability as the full-length HLA-G isoform to inhibit NK and antigen-specific cytotoxic T cell responses. [62]

Table 5: Receptors of HLA-G [85]

Receptor	Cellular distribution	HLA-G-binding site
KIR2DL4 (CD 158d)	NK, T	α1 Domain
ILT-2 (CD85j)[b]	NK, T, B, DC, monocytes and macrophages	α3/β2m domain
ILT-4 (CD85b)[b]	DC, monocytes and macrophages	α3
CD8	T, NK	α3
VD160[c]	Endothelial cells	Unknown

Abbreviations: B, B cells; DC, dendritic cells; NK cells, natural killer cells; T, T cells.
a Although ILT-2 is expressed by B cells, interaction with HLA-G was not described in this context.
b HLA-G dimers bind to these receptors with a higher affinity and slower dissociation rates than monomers. Therefore, the inhibitory function of HLA-G bound to ILTs is mostly due to dimers.
c Needs confirmation.

Functions of HLA-G protein isoforms

Through interaction with the above-described receptors, HLA-G has been shown to inhibit all the actors of the anti-tumor response. Membrane-bound HLA-G reduces NK cell-mediated cytolysis, whether HLA-G is the only inhibitory ligand present on the surface of target cells, or is co-expressed with other inhibitory ligands including classical HLA class I antigens and the non-classical HLA-E protein and/or activating ligands like the MHC class I-related chain-A (MICA) . HLAG also protects these target cells from antigen-specific cytotoxic T lymphocyte (CTL) activity either directly by interaction with the above-mentioned inhibitory receptors , or indirectly, by inhibiting the proliferative response of CD4+ T lymphocytes , which thus leads to the decrease of the cooperation between CD4+ with CD8+ T cells. [62, 86]

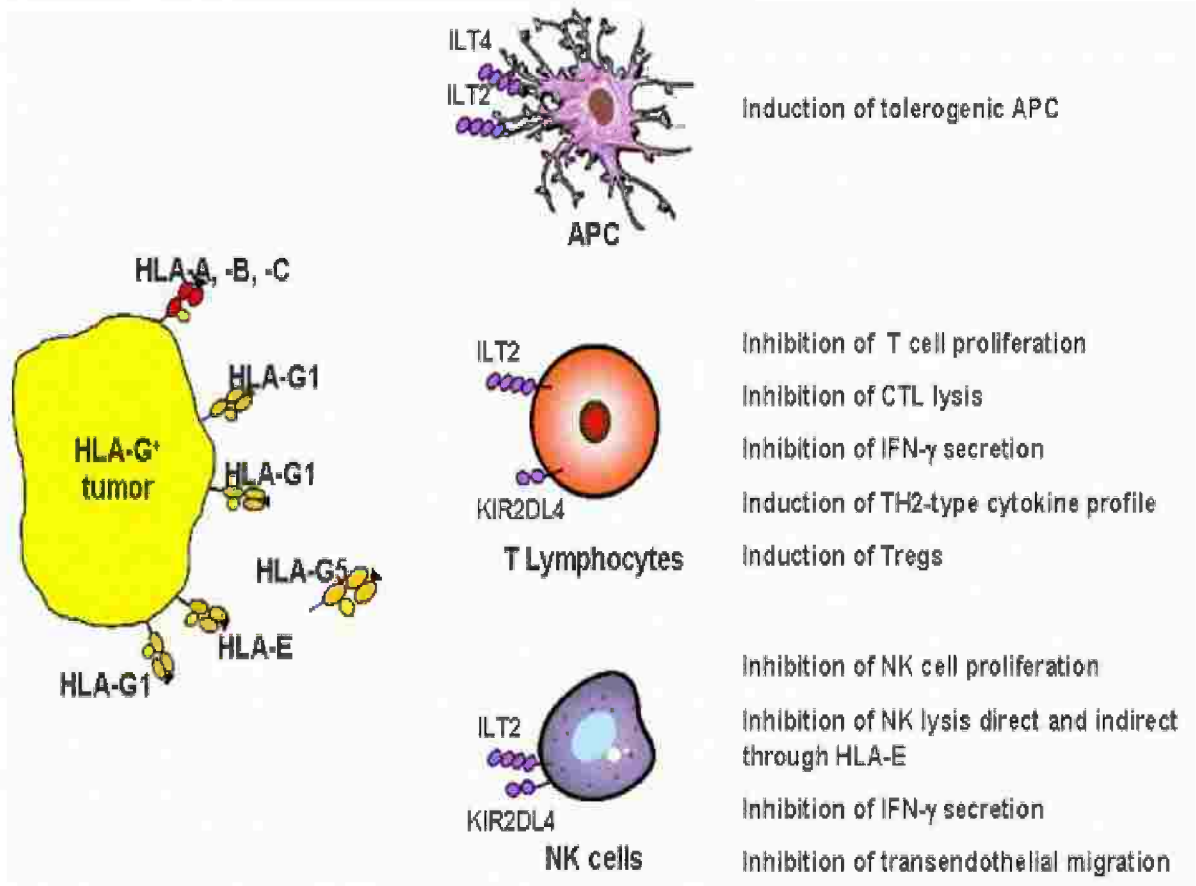


Figure 11: HLA-G properties towards immune cells

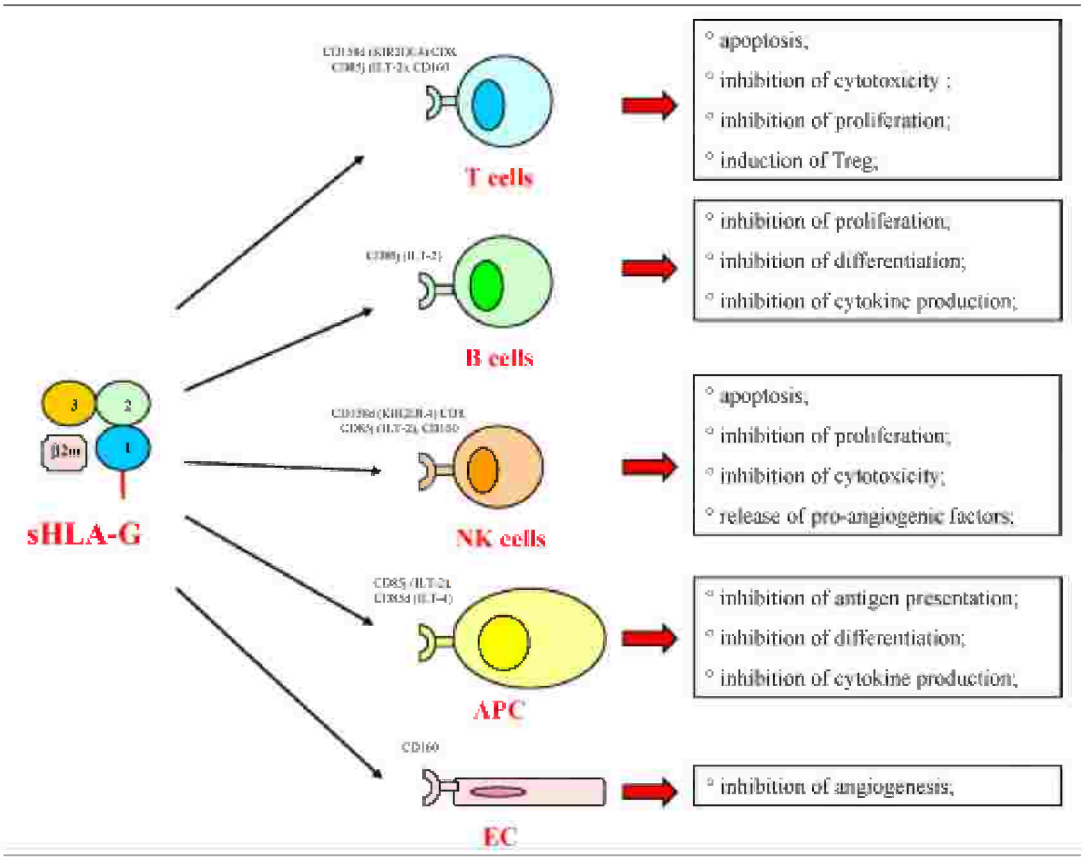


Figure 12: Immunoregulatory activities mediated by sHLA-G. Target cells and receptors involved are also indicated.(87)

APC= Antigen presenting cells, B= B lymphocytes, EC= Embryonic cells, NK= Natural killers, T= T lymphocytes.

HLA-G1 is also able to exert a direct suppressive effect on CD4+ T cells. Furthermore, HLA-G1- expressing antigen presenting cells (APC) render CD4+ T cells anergic and the pre-sensitization of CD4+ T cells by HLA-G1+APC confer them immunosuppressive properties. Recently, another mechanism inducing suppressor T or NK cells has been highlighted. These properties are acquired temporary through the rapid transfer of membrane patches (termed trogocytosis) containing HLA-G, from APC or tumour cells to T or NK cells. Finally, cytokine mediated effects represent a means by which HLA-G can exert immunosuppression. In this regard, HLA-G influences the balance of Th (T helper)1/Th2 cytokines secretion by rather promoting Th2 type responses . Like their membrane-bound counterparts, soluble HLA-G proteins (sHLA-G) have immunosuppressive properties through similar

mechanisms, but with distinct characteristics. Soluble HLA-G antigens have been shown to inhibit NK cell-mediated cytotoxicity. Recently, following interactions between sHLAG and the KIR2DL4 receptor on resting NK cell-surface, NK cells were shown to be activated and to release a set of chemokines and cytokines driving a proinflammatory / proangiogenic response. [62]

An opposite effect on angiogenesis has been observed by inducing endothelial cell apoptosis upon their binding to the CD160 (By55) receptor. Like soluble HLA class I antigens, through upregulation of FasL following the interactions of sHLA-G with CD8, activated T lymphocytes and NK cells come into apoptosis. Moreover, sHLA-G inhibit the cytotoxic activity of antigen-specific CTL, They also decrease CD4+ and CD8+ T cell alloproliferation, by blocking cell cycle progression. Like its membrane-bound counterpart, naive T cells pre-sensitized by HLA-G5 differentiate into suppressor T-cells. These suppressor T cells are not conventional regulatory T cells. Indeed, they express lower CD4 and CD8 antigens, which belong to the TcR/CD3 complex. Such down modulated co-receptors T cells are hyporesponsive to allogeneic stimulus. Lastly, a particular property of sHLA-G is their ability to induce tolerogeneic dendritic cells associated with inhibition of their differentiation. It was shown that HLA-G-expressing melanoma cell lines could release exosomes which bear HLA-G together with well-described proteins as Lamp-2. The secretion of exosomes was shown to be another way for tumour to suppress immune responses. Whether these HLA-G+ exosomes are immunosuppressive remains to be determined.[62]

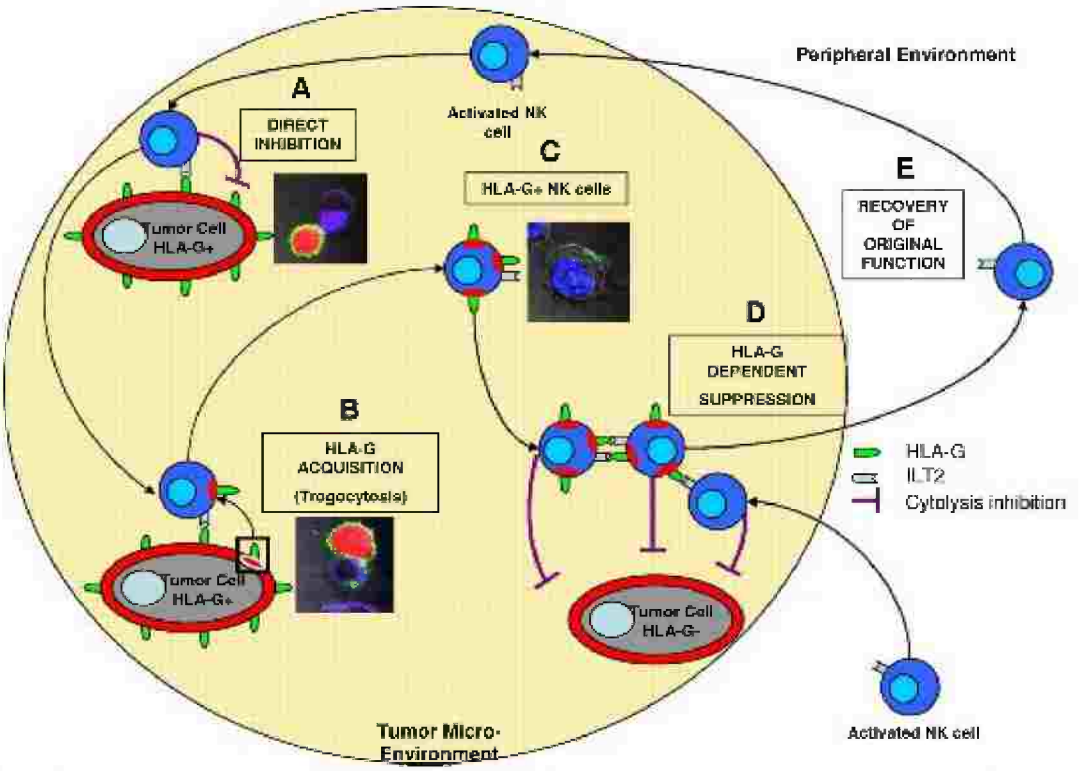


Figure 13: Emergency immune suppression: a possible impact of HLA-G trogocytosis on immune responses. (76)

Factors that enhance expression of HLA-G

Ectopic expression of HLA-G in damaged cells and tissues is upregulated by microenvironmental factors, including stress, nutrient deprivation, hypoxia, hormones such as progesterone, and cytokines such as GM-CSF, IFNs, IL-10, TNFα, TNFβ, and LIF. These factors regulate HLA-G expression at either the transcriptional or posttranscriptional level. However, in cells in which the HLA-G gene is repressed, only cis – acting epigenetic changes, such as DNA demethylating and histone acetylation inhibition, might reactivate HLA-G gene and protein expression. (85)

In the specific context of malignant diseases, these results have to be considered in therapies with DNA- demethylating drugs, such as decitabine or an IFN – based immunotherapy that might induce HLA-G expression, thereby causing an adverse suppressive effect contributing to tumor escape. (85)

Clinical applications

Possible therapeutic applications of HLA-G have been proposed in a range of conditions as a result of the premise that expression of HLA-G is widespread. The use of HLA-G as some sort of 'immune modulator' in organ transplantation, cancer therapy, implantation failure and other diseases seems premature until expression in tissues other than trophoblast cells is confirmed experimentally. ELISAs are already being commercially marketed specifically for the detection of soluble HLA-G in preimplantation embryo supernatants. These tests to select embryos for transfer are now available in clinics offering in vitro fertilization (IVF) even though there is still no definitive evidence that soluble HLA-G is produced by preimplantation embryos or any functional data suggesting how this aids the process of implantation.[88]

HLA-G induces allograft acceptance

During the 1990s, HLA-G was shown to play a role in maternal-fetal tolerance, which constitutes the perfect example of successful physiological immunotolerance of semiallografts. It was hypothesized that HLA-G might also contribute to tolerance in human allotransplantation. To date, the expression of HLA-G was studied in more than a thousand plasma samples from patients after heart, kidney, liver, and liver-kidney transplantation. Expression of HLA-G was detected in situ in cells that are the primary targets of the immune system in rejection, namely, the endomyocardial cells from the heart transplants, the biliary epithelial cells, and the tubular epithelial cells in combined liver-kidney transplants. The results obtained show that patients expressing HLA-G in the graft exhibit significantly better graft acceptance. In addition to determining the HLA-G status of patients via staining of biopsies obtained from organ transplants, measuring sHLA-G plasma levels in transplant recipients represents a strategy by which to monitor the degree of allograft rejection. In this regard, increased HLAG plasma levels were detected in patients with a reduced incidence of acute and chronic rejection after heart and combined liver-kidney transplantation. Furthermore, kidney-transplanted patients who express HLA-G had a lower production of HLA alloreactive antibodies; these antibodies are mainly

involved in acute and chronic graft rejection. These data strongly suggest that HLA-G induced tolerance is also achieved by acting on the B cell response, more specifically by downmodulating antibody production against HLA alloantigens. [85]

Regarding factors that regulate HLA-G expression in patients, sHLA-G plasma levels were found to increase soon after administration of the immunosuppressive drugs cyclosporine and tacrolimus in some heart transplant patients. The influence of the immunosuppressive therapy on HLA-G expression is also supported by preliminary results showing augmented HLA-G plasma levels in kidney transplant patients treated with the CTLA4-Ig fusion protein (Belatacept). This therapeutic agent promotes better graft function and survival. It would be of particular interest to define in future studies the mechanism by which such therapy promotes HLA-G expression. In addition to clinical correlation, the direct involvement of HLA-G in inducing in vivo graft acceptance after human transplantation was provided by the following observations. First, peripheral mononuclear cells (PBMCs) from transplanted patients that have been exposed to high levels of circulating HLA-G do not respond to allogenic stimulus. Second, HLA-G5 purified from plasma of HLA-G+ transplanted patients suppresses T cell alloproliferation in vitro. Finally, peripheral blood mononuclear cell (PBMC) from HLA-G + transplanted patients have immunosuppressive properties and contain an increased proportion of CD3+ CD4low and CD3+ CD8low T cells. Notably, a correlation was clearly obtained between graft survival and HLA-G-related immunological parameters such as IL-10, soluble CD4 or CD8, and CD3+ CD4low or CD3+ CD8low suppressor T cells, which all might contribute to tolerance in HLA-G+ Transplanted patients. In the field of transplantation, the use of human mesenchymal stem cells (MSCs) has generated increasing interest not only because of their ability to undergo differentiation toward cells of different lineages, but also because of their immunosuppressive properties. Thus, adult bone marrow-derived MSCs could be useful for preventing detrimental allogeneic reactions. In this context, adult MSCs were recently described as secreting the sHLA-G5 isoform. Blocking such HLA-G5 molecules led to a reversal in the ability of MSCs[85]

(i) to support in vitro expansion of CD4+ CD25+ FoxP3+ suppressor regulatory T (Treg) cells,

(ii) to inhibit T cell alloproliferation, and

(iii) to suppress NK-mediated cytolysis.

These results show that HLA-G molecules play a major role in the ability of MSCs to modulate immune responses and have important clinical implications in immunosuppressive therapies based on MSC injection in allotransplantation and in regenerative medicine. [85]

A major risk of transplantation is rejection of the transplanted organ by the host immune system. In principle, sHLA-G may help reverse rejection by blocking CTL and NK cell-mediated mechanisms. Studies carried out in transplant recipients have made the following observations:

(i) In renal allograft recipients, the presence of serum sHLA-G is positively correlated with functioning transplants,

(ii) Heart transplanted patients displaying a significant increase in serum sHLA-G in the first month after transplantation have a lower incidence of severe rejection episodes than patients with low levels of the molecule and

(iii) In liver transplanted patients, high serum levels of sHLA-G showed a positive correlation with normal liver function tests, whereas a fall in sHLA-G levels was rapidly followed by deterioration of liver functional parameters.

Taken together, these results suggest that patients showing increased serum levels of sHLA-G shortly after transplantation have lower incidence of rejection episodes likely due to the immunosuppressive effects of sHLA-G. Additional studies are needed to assess whether sHLA-G levels may have a prognostic value in solid organ transplantation. [87]

HLA-G and Immune-mediated disorders

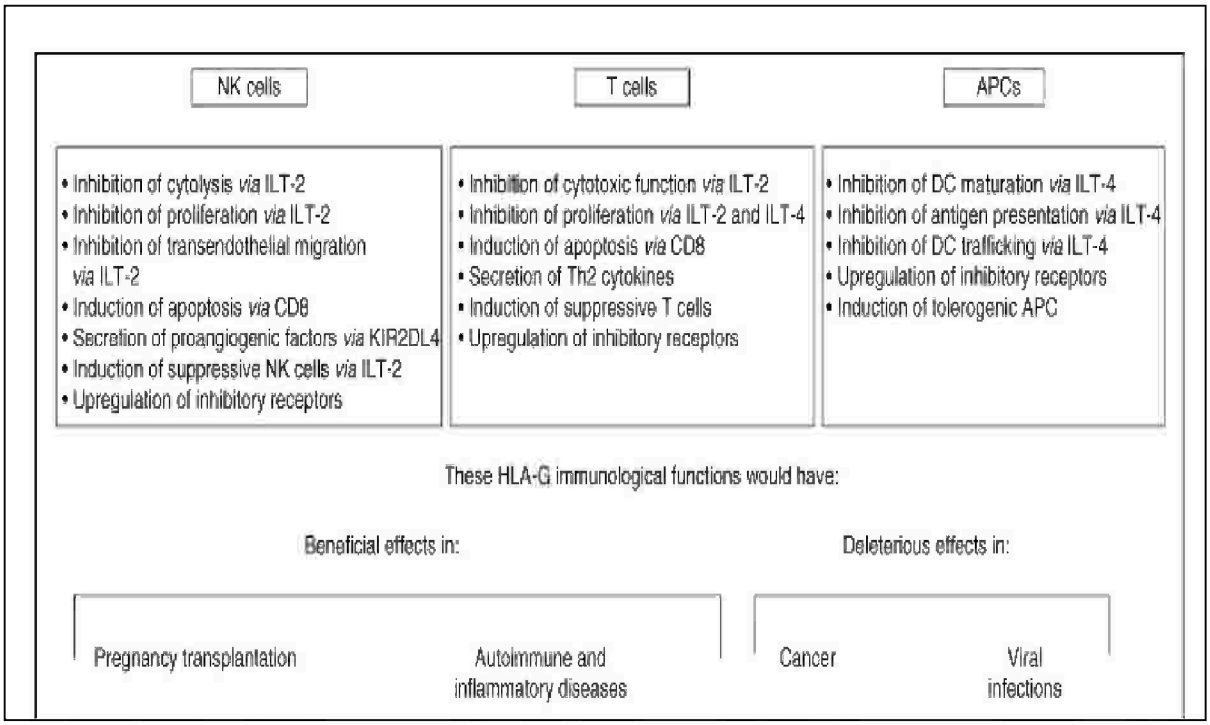
Both HLA-Gand the CD85d (ILT-2) receptor were detected by immunohistochemistry in acute and chronic plaques, perilesional areas and normal white matter of MS patients. sHLA-G levels in CSF were significantly elevated in MS patients with clinically and MRI stable disease. Furthermore, while sHLA-I serum levels were low in clinically active MS patients, sHLA-G serum levels were decreased in clinically stable MS patients. Thus, sHLA-I and sHLA-G1 display opposite trends in relation to disease activity in MS patients. In another study, the same group of investigators correlated sHLA-G levels in the CSF from MS patients with the clinical course of the disease and the results of MRI imaging in patients with RR, SP and PP MS. sHLA-G levels in CSF were significantly increased in clinically stable and MRI inactive individuals, indicating that sHLA-G-mediated immunosuppression may be involved in disease stabilization. Furthermore, sHLA-G and IL-10 levels in the CSF from patients with RR MS were correlated with each other and were found to be increased in MS patients without lesional activity on MRI scans, suggesting the involvement of both molecules in disease remission. [87]

sHLA-G serum levels were significantly decreased in a cohort of patients with rheumatoid arthritis (RA). Furthermore, sHLA-G levels in RA patients positively correlated with parameters of disease activity and presence of HLADRB1 associated epitopes. The low levels of sHLA-G detected in RA patients suggest that T and NK cell activation are not adequately downregulated by sHLA-G molecules.

sHLAG levels were significantly higher in children with atopic asthmatics patients than in healthy controls.

HLA-G expression was detected also in intestinal biopsies and sera from patients with celiac disease. This finding may suggest that HLA-G expression reflects an attempt to restore tolerance to gluten and counterbalance inflammation. [87]

Table 6: Immunological properties of HLA-G: implications in human diseases.



HLA-G and infectious diseases

In a similar fashion to tumors, viruses develop strategies to escape the host immune reaction. Because the induction of HLA-G expression by virus-infected cells might be an additional mechanism that helps viruses to subvert host defences, investigations were conducted regarding the role of HLA-G after infections with the cytomegalovirus (CMV), human immunodeficiency virus (HIV), and neurotropic viruses. In HIV-infected patients, HLA-G expression was upregulated in CD8+ T cells and monocytes. Whereas, long-term progressor patients expressed high plasma levels of HLA-G, the long-term non progressor patients did not. This suggests a new mechanism employed by HIV to evade the cytotoxicity of immune cells. The role of HLA-G in HIV infection was further supported by the association between HLA-G genetic variants with susceptibility to HIV. Moreover, sHLA-G is induced in CMV-infected monocytes. Analysis of HLA-G expression in neurotropic viral infections showed that herpes simplex virus type 1 and rabies virus upregulate the neuronal expression of HLA-G isoforms both in infected cells and neighboring uninfected

cells. HLA-G might be involved in their escape from the immune response in the nervous system. [87]

Finally, a recent report showed increased HLA-G5 plasma levels in patients with septic shock. Such high HLA-G levels allow for the prediction of survival better than HLA-DR detection on monocytes. These data strongly suggest that HLA-G measurement in plasma might replace currently used HLA-DR as a prognostic marker after sepsis. [85]

HLA-G expression in tumor lesions and malignant effusions

In 1998, a study described a high level of HLA-G in a skin biopsy from melanoma metastasis and in a melanoma cell line for which presence of HLA-G was related to protection from NK lysis. Two following studies on two hundred patients melanoma biopsies revealed that:

(i) HLA-G protein was expressed in thirty percent of the patients ,

(ii) HLA-G expression is associated with malignant transformation of melanocyte as this protein was detected in both primary and metastatic tumour sites, but neither in adjacent tumor tissue or in spontaneous tumour regression site or in healthy skin ,

(iii) Higher levels of inflammatory tumor-infiltrating cells were observed in malignant melanoma lesions in comparison to begnin ones and

(iv) Upregulation of HLA-G in melanocytes is a better predictor of malignancy than classical HLA class I antigens defects, which are often described as an important mechanism of tumor escape from immunosurveillance. This association between the presence of HLA-G and the malignant nature of the tumor suggested that HLA-G was a mechanism for tumors to escape immune surveillance. [89, 90]

Following this description, HLA-G expression in melanoma lesions was confirmed by other groups and numerous other tumor lesion types of either

ectodermic or mesodermic or endodermic origin. Today, in about two thousand patients analyzed, HLA-G protein was found in almost all types of cancer whatever their origin, but in varying proportions ranging between 10% (acute leukemia) to 95% (esophageal squamous cell carcinoma). [62]

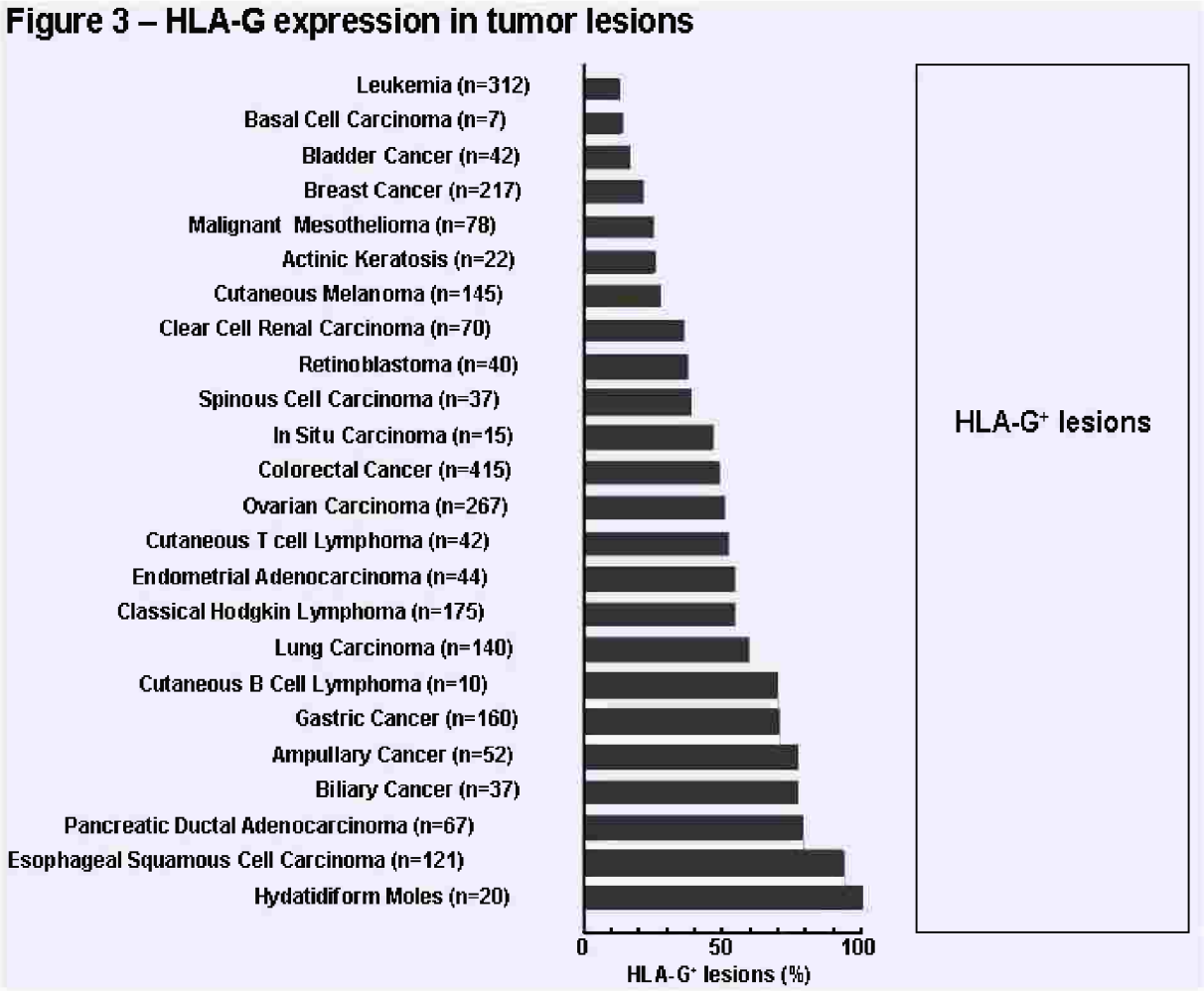


Figure 14: HLA-G expression in tumor lesions

Since that review, among 140 patients with lung cancer analyzed, approximately 60% of them expressed HLA-G protein. Two other types of cancers were studied: in 175 patients with classical Hodgkin lymphoma, HLA-G protein was found in 50% of cases and in 121 patients with esophageal squamous cell carcinoma, this proportion reached 95%. This heterogeneity may reflect the differences in the biology of individual tumors, in study design and/or the sensitivity of the methods

used to detect HLA-G protein in malignant lesions. HLA-G expression is also heterogeneous within a tumor type and within individual lesions. Indeed, HLA-G has been detected in the tumor tissue and/or in the infiltrating lymphocytes in tumors such as melanoma, breast cancer, and lung carcinoma. Moreover, HLA-G expression can concern different number of cells within a type of tumor according to the patients. Through the recent development of HLA-G-specific ELISA, high levels of HLA-G in its soluble form have also been detected in the plasma of patients with various cancers, including melanoma, glioma, multiple myeloma, lymphoblastic and monocytic acute leukemia, neuroblastoma , and in ascites from breast and ovarian carcinomas . [51]

Regulation of HLA-G expression in cancer

Up to date, in more than two hundred cell lines derived from malignant tumor biopsies, HLA-G protein was only detected in about ten, what contrasts with the proportion of surgically removed malignant tumor lesions which express HLA-G. This discrepancy shows that in vitro, factors which were maintaining the expression of HLA-G are not present anymore, and that in vivo, HLA-G expression is activated by environmental stimuli such as stress conditions, cytokines and epigenetic variations. Indeed, HLA-G is a stress-inducible gene. Heat shock and arsenite induced an increase of the different HLA-G alternative transcripts without affecting the other MHC class I HLA-A, -B, -C, -E and -F transcripts in melanoma cells. A stress situation is represented by hypoxia, which exists in the surrounding microenvironment of rapidly growing tumors. Hypoxia was shown to induce the hypoxia-inducible factor-1a (HIF-1a) which can in turn, trigger the transcription of HLA-G gene in HLA G-tumor cells. In contrast, HLA-G expression was decreased in HLAG+ tumor cells. In both up- and down-modulation, HLA-G expression depends on HIF-1a stabilization.[51, 58]

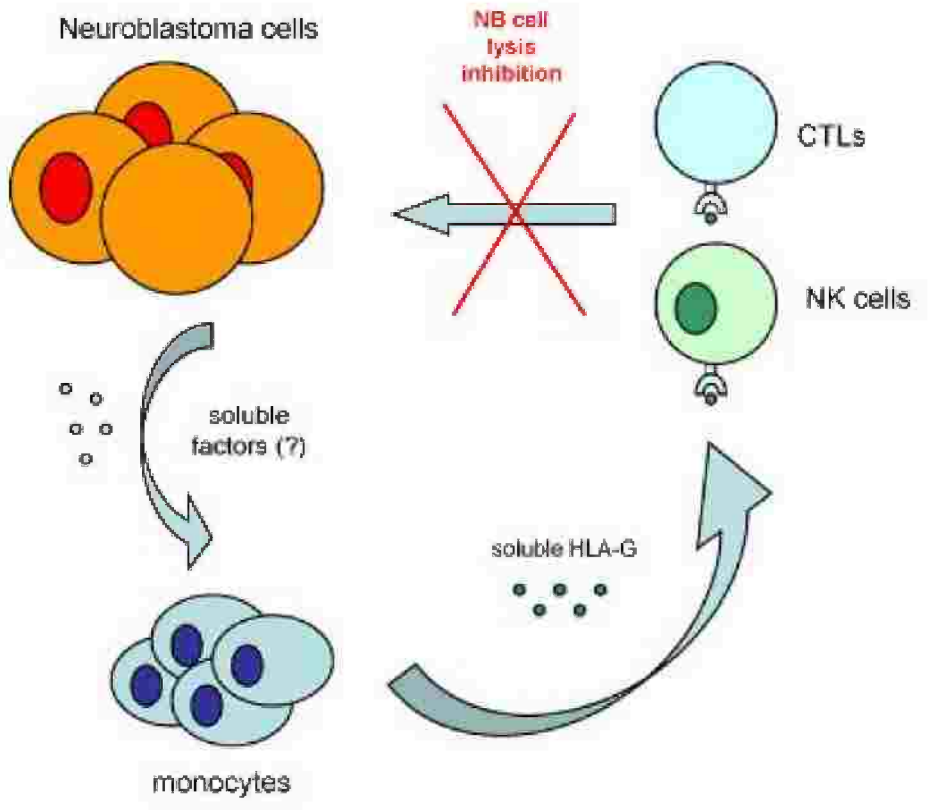


Figure 15: Schematic representation of a novel mechanism utilized by human neuroblastoma cells to elude the control of the host's immune system.

Finally, it is well-known that both the depletion of the essential amino acid tryptophan and the accumulation of tryptophan metabolites from the microenvironment provoke an inhibition of immune cells function. This represents another tumor escape mechanism from immune system as some tumors or APC in tumor-draining lymph nodes express the indolamine 2,3-dioxygenase (IDO), an enzyme which metabolizes tryptophan .

A link between HLA-G and IDO molecules was found that:

(i) Inhibiting the function of IDO up-regulates HLAG1 cell-surface expression on APC and tumor cell lines

(ii) IDO induces HLA-G expression during monocyte differentiation into dendritic cells , and

(iii) IDO and HLA-G inhibit T cell alloproliferation through two independent but complementary pathways. [62]

Thus, the recent development of IDO inhibitors as a new immunoregulatory treatment modality for clinical trials has to consider the possible stimulation of HLA-G expression. Numerous studies have investigated the cytokine - mediated induction of HLA-G expression. Particularly, the anti-inflammatory and immunosuppressive IL-10 cytokine secretion by lung carcinoma cells and T and B lymphoma cells has been correlated with concomitant HLA-G expression. Transactivation of HLA-G transcription has also been demonstrated by JEG3 choriocarcinoma cell after exposed to leukemia inhibitory factor (LIF). Furthermore, interferon (IFN)-a, -β and -γ enhance HLA-G cell-surface expression by tumors or monocytes. This up-regulation of HLA-G at the tumor site represents one potential side effect of the administration of IFN for immunotherapy and may confer immunoprotection to tumor cells, thus favoring tumor expansion. In this regard, an association has been established between the lack of clinical response to therapy with IFN-a high doses and HLAG expression in melanoma lesions. The expression of inflammatory cytokines such as IFN-γ is under the control of the nuclear factor- κ B (NF-κB), a pivotal transcription factor of innate and adaptative immunity. NF-κB activation enhances HLA-G intracytoplasmic tumor cell content, but promotes the proteolytic shedding of membrane-bound HLA-G. Moreover, HLA-G1+ tumor cells activate NF-κB in NK cells. This activation occurs through interactions between the a1 domain of HLA-G and presumably the KIR2DL4 receptor.[51, 62]

Remarkably, cytokines have no effect on HLA-G gene transcription in tumor cells in which this gene is repressed. This led to propose the hypothesis of the existence of epigenetic mechanisms, which may activate the HLA-G gene in some tumors. Global genomic hypomethylation occurs frequently during carcinogenesis and genetic lesions in methyl chromatin related genes, such as histone deacetylase, are supposed to influence the epigenetic alterations involved in cancer. Exposure of some tumors cell lines to histone deacetylase inhibitors, or the decitabine DNA demethylating agent, reactivate both HLA-G gene transcription and transduction. Thus, the HLA-G gene depression must be considered as adverse effect following

chemotherapy with drugs such as decitabine, which are currently used to reactivate tumor suppressor genes and other genes involved in invasion and metastasis. [62]

Table 7: Summary of the human diseases associated with HLA-G: roles and clinical applications[85]

Physiopathology	**Expression and biological functions**	**Clinical Applications**
Pregnancy	Protection of the fetus from uterine NK cell cytolysis Secretion of cytokines and angiogenic factors by uterine NK cells Embryo implantation and placental development Reduced expression in pregnancy disorders associated with fetal loss (preeclampsia, recurrent spontaneous abortion)	Marker of efficient implantation after IVF
Transplantation	Neo-expression in grafted organs and increased plasma levels in heart, kidney, liver, or liver-kidney transplant patients Positive association with better graft function and survival Negative association with HLA alloantibody titers in patients Positive correlation with high plasma levels of IL-10, sCD4,and sCD8 and overrepresentation of peripheral blood CD3+ CD4low and CD3+ CD8low T cells Induction of allograft acceptance through inhibition cell alloresponses and induction of CD3 and CD3 + CD8 low suppressor T cells and tolerogenic dendritic cells Constitutive expression by human MSCs and contribution to their immunosuppressive properties	Marker of clinical follow-up to select patients expected to better accept their allograft and to benefit from reduced immunosuppressive treatment Therapeutic use of HLA-G, derivatives, HLA-G inducers, or MSCs to prevent allograft rejection
Inflammatory and	Neo-expression in digestive tract	Differential diagnostic tool between

autoimmune disorders	diseases (coeliac disease, ulcerative colitis) Neo-expression in cutaneous diseases (psoriasis, atopic dermatitis) Reduced plasma levels and lower expression by monocytes in MS patients	ulcerative colitis and Crohn's disease Marker of clinical follow-up negatively associated with disease activity in MS Marker of IFN treatment efficacy in MS
Cancer	Neo-expression in malignant lesions and increased plasma levels in cancer patients associated with bad clinical evolution Tumor-driven escape mechanism inhibiting antitumor responses	Marker of clinical follow-up positively associated with aggressiveness and poor prognosis in ovarian cancers, B-CLL, and in gastric and colorectal tumors Differential diagnostic tool by which to identify trophoblastic tumors Target for antitumor therapy by blocking expression (RNA interference) or function (neutralizing antibodies)
Infectious diseases	Expression after infections with CMV, HIV, and neurotropic viruses Increased plasma levels in patients with septic shock	Predictive marker of survival in patients with septic shock
Abbreviations: B-CLL, B cell chronic lymphocytic leukemia; CMV, cytomegalovirus; HIV, human immunodeficiency virus; IVF, in vitro fertilization; MS, multiple sclerosis.		

HLA-G in cancer immunediting

During the elimination phase which matches with cancer immunosurveillance, classical HLA class I expression at tumor cell-surface is supposed to be unchanged. Tumor -infiltrating lymphocytes and NK cells produce Th1-type cytokines. IFN-gamma is one of the cytokines up-regulating HLA-G expression in tumor cells either directly or indirectly through induction of IDO. Towards its predominant inhibitory role, HLA-G could greatly weaken host anti-tumoral immune responses. During the equilibrium phase which corresponds to cancer persistence, epigenetic changes take place frequently and contribute to the development of non-immunogenic tumor cell clones. At present, in vitro studies showed that demethylation and histone deacetylation reverses HLA-G gene silencing. In addition to its direct inhibitory role on immune cells, HLA-G protein may also play this role indirectly through the plasma

membrane stabilization of the non-classical MHC class I HLA-E protein. HLA-E reaches cell surface through binding of MHC class I leader peptide. [62]

Although classical HLA class I molecules can be completely lost, HLA-G can mediate the membrane expression of HLA-E, which confers additional protection of tumor cells to NK cytolysis. Moreover, HLA-G also contributes to the already altered antigen presentation by down modulating HLA class II molecules on APC. The escape phase in which cancer progresses, is the phase where HLA-G is preferentially expressed comparatively to initial malignant tumor lesions. Tumors generate an appropriate microenvironment that allows them to prevent their immune cell elimination, thus favoring their growth. The mechanisms to achieve this goal include the modulation of antigen expression that allows preventing activation of the immune system, the induction of peripheral tolerance by induction of anergy or induction of immunosuppressor cells, and the production of immunosuppressive cytokines. HLA-G plays a significant role in these mechanisms because HLA-G remains the almost single-molecule expressed by tumors. In addition to local effects at its site of expression, secreted soluble HLA-G could also have systemic inhibitory activity through its distribution in blood circulation. Among the immunosuppressive cytokines produced by tumors, IL-10 is responsible for HLA-G up-regulation in cancer. Both IL-10 and HLA-G may be produced by tumor cells themselves or by tumor-infiltrating cells. There is an amplification loop since both IL-10-induced decrease of the production of Th1-type cytokine and HLA-G expression are able to increase IL-10 production. Moreover, HLA-G was shown to increase its own inhibitory receptors in NK, APC and T effector cells, and IL-10 can also modulate the KIR repertoire on NK and T cells, what further contribute to dampen immune responses. During this phase, chronic inflammation is assumed to favor tumor growth through activation of NF-κB, which may enhance systemic inhibitory action through the release of soluble HLAG1 from proteolytic shedding of membrane HLA-G1. The rapid tumor cell proliferation creates a hypoxic microenvironment, a stress condition which also promotes tumor invasion per se but in addition, by inducing HLA-G expression. Importantly, some therapeutic strategies, as either immunotherapy using IFN-α, or chemotherapy with DNA - demethylating or histone deacetylating agents, or

therapeutic vaccination using IDO inhibitors, must be revised since these treatments were shown to upregulate HLA-G. [62]

Biological relevance of HLA-G expression in cancer

In vitro studies have shown that HLA-G-endogenously expressing melanoma, glioma and renal carcinoma cell lines are protected from lysis by alloreactive NK and lymphokine activated killer cells and/or antigen-specific CD8+ T cells. This protective effect was directly due to HLA-G expression by tumor cells since the blockade of this molecule restored the cytotoxic activity of effector cells. Since then, two studies have reinforced the role of HLA-G in tumors. In the first study, they derived a HLA-G+ melanoma cell line (Fon), from a HLA-G+ melanoma biopsy. The Fon cell line expressed high levels of membrane-bound HLA-G1, which confers resistance to NK cell line lysis through interaction with ILT-2 inhibitory receptor. During the long-term spread of Fon cells in culture, the expression of HLA-G1 has been lost, as was its protection against the NK cell cytotoxicity. Although IFN-β, -γ or decitabine treatments enhanced HLAG1 expression in the primary Fon cells, neither these cytokines, nor this DNA-demethylating drug brought back HLA-G1 transcription. Altogether, these results emphasize the difficulty using cell lines derived from tumor biopsies to establish the physiopathological relevance of HLA-G in anti-tumor responses since HLA-G expression can be lost during long-term tumor expansion. Moreover, they support the role of HLA-G1 expressed at tumor cell surface in preventing host innate immune cell responses. As described for other tumor type, the second study showed that neuroblastoma patients had significant higher serum levels of sHLA-G than healthy donors. The source of sHLA-G in neuroblastoma patients was not tumor cells but monocytes in an activated state.[62]

The sHLA-G produced was able to inhibit CTL and NK cell-mediated cytotoxicity against tumor cells. Interestingly, the sHLA-G-secreting monocytes were instructed by neuroblastoma cells through the release of soluble factors that may be

IL-10 or TGF-s1. These monocytes display features of macrophage-like activated cells but shift towards a more anergic phenotype since they secrete higher levels of immunosuppressive sHLA-G and lower IL-12, a cytokine which promotes anti-tumor responses. These findings support an in vivo systemic effect of HLA-G in cancer, and provide guidance on a novel mechanism of tumor immune evasion. [62]

Clinical significance of HLA-G expression in cancer

As above described, HLA-G expression has always been detected in malignant tissues or effusions and has never been found neither in healthy tumor surrounded areas, nor in tissues or effusions from patients suffering of begnin disease, nor in the corresponding tissues from healthy individuals . In melanoma, upregulation of HLA-G molecules in melanocytic cells appears as a better predictor of malignancy than classical HLA class I antigen defects frequently observed in this cancer type. [62]

The idea that HLA-G expression could be a prognostic factor has emerged recently. Indeed, HLA-G expression and/or sHLA-G high levels have been significantly correlated with poor prognosis in non - small cell lung cancer, melanoma, glioblastoma, ovarian carcinoma, B-CLL, cutaneous T cell lymphoma, neuroblastoma and digestive cancers. In particular, in ovarian carcinomas, high levels of soluble HLA-G protein were measured in the effusions produced in late-stage disease which overlaps with the first appearance of metastases. In digestive cancers and B-CLL, HLA-G expression was shown as being an independent prognostic factor. In multivariate analysis of B-CLL patients, HLA-G expression was an even better independent prognostic factor than the zeta-associated protein 70 (ZAP-70) or CD38 status. Finally, the role of HLA-G in tumor escape from host immune cell is emphasized by its involvement of the resistance to IFN therapy observed in some melanoma patients. [62]

Finally, human leukocyte antigen G (HLA-G) molecule exerts multiple immunoregulatory functions that have been suggested to contribute to the immune

evasion of tumor cells. Studies of soluble HLA-G expression in malignant hematopoietic disease are controversial, and the functions of HLA-G in this context are limited. In AML, soluble HLA-G positive patients have a significant higher bone marrow leukemic blast cell percentage when compared with that of soluble HLA-G negative patients. Total T-cell percentage is dramatically decreased in soluble HLA-G positive **subjects**. [62]