Structural and functional Alterations of FLT3 in Acute Myeloid Leukemia

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Abstract:

Hematopoiesis is highly regulated through cytokine-induced stimulation of multiple signal transduction pathways in order to mediate appropriate differentiation and proliferation of specific progenitor populations. Ligand-induced stimulation of the FMS-like tyrosine kinase 3 (FLT3) leads to activation of multiple downstream effector pathways resulting in differentiation and proliferation of specific progenitor cell populations. Genomic alterations of the FLT3 gene leads to autonomous receptor activation, dysregulation of FLT3 signal transduction pathways, contributes to myeloid pathogenesis, and have been linked to response to therapy and clinical outcome. Exploring the mechanisms by which these FLT3 alterations lead to dysregulated proliferation would provide a better understanding of the molecular pathogenesis of AML and may provide insights into potential therapeutic interventions.

Structure and Function of FLT3

FLT3, a receptor tyrosine kinase (RTK), is a membrane-bound receptor with an intrinsic tyrosine kinase domain. FLT3 is composed of a immunoglobulin-like extracellular ligand-binding domain, a transmembrane domain, a juxtamembrane dimerization domain and a highly conserved intracellular kinase domain interrupted by a kinase insert. FLT3 belongs to the class III subfamily of RTKs which include structurally similar members such as c-FMS, c-KIT and PDGF receptor. FLT3 is primarily expressed on committed myeloid and lymphoid progenitors(1, 2) with variable expression in the more mature monocytic lineage. FLT3 expression has been described in lymphohematopoietic organs such as the liver, spleen, thymus, and placenta. (3, 4)

In the un-stimulated state, FLT3 receptor exists in a monomeric, unphosphorylated form with an inactive kinase moiety. Upon interaction of the receptor with FLT ligand (FL), the receptor undergoes a conformational change, resulting in the unfolding of the receptor and the exposure of the dimerization domain, allowing receptor-receptor dimerization to take place. This receptor dimerization is the prelude to the activation of the tyrosine kinase enzyme, leading to phosphorylation of various sites in the intracellular domain. The activated receptor recruits a number of proteins in the cytoplasm to form a complex of protein-protein interactions in the intracellular domain. SHC proteins, GRB2, GRB2-associated binder 2 (GAB2), SHIP, CBL, and CBLB (CBLB related protein) are a few of the many adaptor proteins that interact with the activated FLT3 receptor **.**(5-10) As each protein binds to the complex, it becomes activated in turn, resulting in a cascade of phosphorylation reactions that culminates in activation of a number of secondary mediators, including MAP kinase, STAT and AKT/PI3 kinase signal transduction pathways. Once activated, these activated mediators are chaperoned to the nuclear interphase by HSP90, where the message is translocated to the nucleus. In the nucleus, these transcriptional mediators trigger a series of events culminating in regulation of cell differentiation, proliferation apoptosis, and cell survival (Figure 1).

FLT3 Function in Normal and Malignant Hematopoiesis:

FLT3 activation regulates a number of cellular process (e.g. phospholipid metabolism, transcription, proliferation, and apoptosis), and through these processes, FLT3 activation plays a critical role in governing normal hematopoiesis and cellular growth.(11, 12) Optimum FLT3 function requires the coordinated effort of other growth factors such as SCF, and IL3.(12, 13) Combinations of FL and other growth factors have been found to promote proliferation of primitive hematopoietic progenitor cells as well as more committed early myeloid and lymphoid precursors.(11, 12, 14, 15) FL stimulation appears to mediate differentiation of the early progenitors, where exposure of the hematopoietic progenitors to FL, leads to monocytic differentiation, without significant proliferation.(12) Although FLT3 knockout mice have a subtle phenotype, (16) mice transplanted with FLT3 knock out cells displayed a more global disruption of hamatopoiesis.(16) In addition, if both KIT and FLT3 were knocked out, mice developed severe, life-limiting hematopoietic deficiencies. Thus, the *in vitro* data and murine knockout models confirm a major role for FLT3 in normal hematopoiesis, especially in times of hematopoietic stress.

Expression of FLT3 has been evaluated in hematologic malignancies. The majority of B-cell ALL and AML blasts (> 90%) express FLT3 at various leves.(1) Although less frequently and with more variable expression levels, FLT3 receptors are also expressed in other hematopoietic malignancies, including myelodysplasia (MDS), chronic myeloid leukemia (CML), T-cell ALL, and chronic lymphocyctic leukemia (CLL).(1) Recent data suggest that very high levels of FLT3-WT receptors *may* promote constitutive activation of the wild-type receptor in malignant cells,(17) and other studies have found that increased FLT3-WT expression in leukemic blasts may be associated with a worse prognosis (18)

Genomic Alteration of FLT3 in AML

Early studies evaluating function altering genomic alterations in FLT3 gene identified 2 distinct mutations in juxtamembrand and kinase domains of the FLT3 gene. Internal tandem duplication of the juxtamembrane domain coding sequence in FLT3 (FLT3/ITD) was identified in a high proportion of patients with AML.(19) Additional studies identified missence mutations in the activation loop of the tyrosine kinase domain of FLT3 (FLT3 Activation loop mutation, FLT3/ALM).(20)

<u>FLT3 internal tandem duplications (FLT3/ITD):</u> These mutations are the result of a segmental duplication of a fragment within the juxtamembrane domain coding region (encoded by exons 14 and 15) of FLT3, and are the most common type mutation in hematologic malignancies, occurring in CML (5-10%), MDS (5 – 10%), and AML (15 – 35%) patients.(19, 21-29) Prevalence of FLT3/ITD is highly age dependent, where it is rare in infant AML, and the prevalence increases in a step-wise manner to 5-10% in age 5-10, 20% in young adults and

>35% in AML patients older than 55 years. (28) There is also considerable variability in size, and region of ITD involvement (ranging from 3 to >400 basepairs).(19, 30, 31)

In vitro studies have demonstrated that FLT3/ITDs promote ligand-independent receptor dimerization, leading to autonomous phosphoyrylation and constitutive activation of the receptor, culminating in cytokine independent cellular proliferation.(21, 32-35) The specific mechanism by which FLT3/ITDs lead to auto-dimerization is unknown, however, JM domain is thought to act as a negative regulatory domain by preventing activation loop from adopting active conformation, thus maintaining the receptor in an auto-inhibited state. Three dimensional structure analysis of the FLT3 receptor suggests that segmental duplication of the JM domain may disrupt steric hindrance that normally prevent auto-dimerization, allowing receptor-receptor interaction to take place. Similar "repulsive" forces have been described in JM domains of other receptors (e.g. KIT), and disruption of the JM domain in other receptors also promotes constitutive activation.(36, 37) Most likely, the dimerization of the FLT3/ITD receptors causes additional structural changes in the receptor to a more relaxed confirmation, exposing the phosphoryl acceptor sites within the TKDs and promoting autophosphorylation.(38)

FLT3/ITDs are thought to promote proliferation *via* the activation of multiple signaling pathways including RAS/MAPK, STAT and the AKT/PI3 kinase pathways. Mizuki et al. demonstrated that cytokine independent cellular proliferation of FLT3/ITD transduced cells was mediated by RAS and STAT5 pathways.(39) Demonstration of STAT5 activation in FLT3/ITD highlighted that some of the effects of FLT3/ITDs are unique to the mutated receptor, where in contrast to FLT3/ITD, ligand-induced FLT3-WT activation does not lead to STAT5 activation and no STAT5 DNA binding.(34) Subsequent studies have determined that codons T589 and T591 within the JM of the mutated FLT3 receptor may account for some of the STAT5 activation in cells harboring FLT3/ITDs.(40) Other studies have demonstrated that in addition to RAS and STAT pathways, FLT3/ITDs constitutively phosphorylate and activate AKT, and that ligand independent growth requires this aberrant AKT activation by FLT3/ITDs, such that the ligand independent growth is reversed if AKT activation is blocked.(41)

<u>FLT3 Activation Loop Mutations (FLT3/ALM):</u> Missense point mutations within the activation loop of the FLT3 kinase domain (FLT3/ALM) are the second type of FLT3 mutation.(20, 25, 28, 42-45) These mutations are also referred to as FLT3 tyrosine kinase domain mutations (FLT3/TKD). FLT3/ALMs have been found in the malignant cells from patients with CML (~1%), ALL (1-3%), MDS (2-5%), and AML (5-10%).(20, 25, 28, 46) Since patients seldom harbor both a FLT3/ITD and FLT3/ALM,(47) a high percentage of AML patients (25 – 45%) will have at least one type of FLT3 mutation, making FLT3 mutations one of the most common genetic abnormalities in AML.(25, 28, 43, 48) In contrast to the age-dependent increase in the prevalence of FLT3/ITD, FLT3/ALM prevalence appears to be constant across all age ranges.(28) The majority

of the FLT3/ALMs occur in codons 835 with a change of an aspartic acid to tyrosine (D835Y), however, other point mutations, deletions, and insertions within codon D835 and its surrounding codons have been described.(20, 25, 44, 45, 49)

Although FLT3/ALM promote autophosphorylation of the receptor, constitutive receptor activation, and ligand-independent proliferation, similar to that of FLT3/ITD, (20, 21, 32-34, 49) there are significant biological differences between the two types of FLT3 mutations.(40, 50-54) FLT3/ITDs and FLT3/ALMs appear to promote activation of different downstream effectors,(50) and different biological responses. Animal studies have demonstrated that mice harboring FLT3/ITDs primarily develop an oligoclonal myeloproliferative disorder, while mice harboring FLT3/ALM are more likely to develop oligoclonal lymphoid disorders.(51)

Clinical Significance of FLT3 Mutations

Identification of FLT3 mutations in AML has yielded novel approaches to the management of this disease. Whether it is through their utility as prognostic factors or their use as a target for directed therapies, FLT3 mutations have provided clinicians with novel therapeutic options for a large subset of AML patients. We will specifically address the prognostic significance of the FLT3 mutations and their role in risk-based therapy, including hematopoietic cell transplantation (HCT) for AML patients with FLT3 mutations. Furthermore, we will review the available data on the efficacy in AML patients of small molecule inhibitors against FLT3 mutations.

Prognostic Significance of FLT3/ITD: Kiyoi et al. described FLT3/ITDs in AML, finding that approximately 25% of AML patients harbored these mutations.(26) In their study, AML patients with FLT3/ITDs had a higher diagnostic WBC, and the FLT3/ITDs were associated with normal cytogenetics. Although there was no significant difference in complete response (CR) rates between patients with and without FLT3/ITDs, those patients with FLT3/ITDs had a significantly higher relapse rate (RR) and worse overall survival (OS).(26) Specifically, in younger adult patients (< 60 years old), FLT3/ITDs was the strongest predictor of outcome.(26) Subsequent larger studies have confirmed that presence of FLT3/ITD is an independent prognostic factor for relapse and poor outcome in AML.(24, 25, 27) Kottaridis et al. examined the prevalence and prognostic significance of FLT3/ITDs in a cohort of over adult 850 patients.(27) These investigators found a FLT3/ITD prevalence of 27%. Their study confirmed that FLT3/ITDs were associated with leukocytosis and normal cytogenetics. In their study, AML patients with FLT3/ITDs had a lower remission rate, higher relapse rate and worse survival. Multivariate analyses controlling for other prognostic factors found that FLT3/ITDs were the most significant prognostic factor with respect to RR and disease free survival (DFS).(27)

Other studies have confirmed the prognostic significance of FLT3/ITD in large adult and pediatric AML trials.(24, 28, 55) In these studies survival for patients with FLT3/ITD was 20-30% compared to 50% for those without FLT3/ITD. They also made an observation that allelic variation (mutant to wild type ratio) observed in patients with FLT3/ITD appeared to influence outcome.(28, 55) Evaluation of various thresholds of FLT3/ITD allelic ratio (ITD-AR) established an allelic ratio threshold that demarcated patients with FLT3/ITD at high risk of relapse from those expected to have a more favorable outcome, demonstrating that it is not merely the presence of FLT3/ITD, but the allelic variation that determines clinical outcome.(28) Similar work in other studies have demonstrated difference in clinical outcome for those with differing allelic ratios.(55, 56)

Clinical Significance of FLT3/ALM: Shortly after FLT3/ITD was recognized, the presence of FLT3 activation loop domain mutations (FLT3-ALM) were reported in 7% of patients with AML and their presence was associated with poor outcome.(20) More thorough evaluation of large cohort of patients have more fully defined the prevalence and prognostic significance of FLT3/ALM in pediatric and adult AML. Prevalence of FLT3/ALM is 6-8%, regardless of age and the presence of FLT3/ALM and FLT3/ITD appears to be mutually exclusive.(28, 57) These studies further demonstrated that presence of FLT3/ALM is not associated with leukocytosis, and in contrast to FLT3/ITD, those with FLT3/ALM do not have adverse outcome. As biologic studies have previously demonstrated functional differences between the FLT3/ITD and FLT3/ALM, such clinical differences between the two types of FLT3 mutations are not surprising.(50, 51) Reviewing the clinical outcome data in the context of FLT3/ITD allelic variation and FLT3/ALM, one would conclude that factors in addition to the presence of FLT3 mutation or activation of the receptor impact biology of the disease and in fact other structural alterations associated with FLT3/ITD may impact disease progression.

Underlying Mechanism of Allelic Variation in FLT3/ITD:

As variation of allelic ratio is associated with clinical outcome, delineation of the underlying mechanism of the allelic variation may provide insight into the pathogenesis of AML and guide therapeutic intervention. Initial evaluation of homozygous FLT3/ITD by STR primers revealed loss of heterozygosity (LOH) as a contributor to the disease pathogenesis in FLT3/ITD-positive AML.(58) A study by Whitman et al. substantiated the presence of LOH in a cohort of FLT3/ITD AML patients, and demonstrated that presence of LOH was associated with high risk disease.(59) Novel high throughput methods have allowed more comprehensive evaluation of the LOH in patients with FLT3/ITD. Whole genome evaluation of copy number and LOH alterations by SNP/CGH array technology demonstrated large segmental LOH of chromosome 13 in a subset of patients with FLT3/ITD (those with high ITD-AR)(60) The region of LOH extended from regions centromeric to FLT3 and included the entire 13q telomeric to FLT3. This large region of 13qLOH was limited to FLT3/ITD samples and was not observed in those with FLT3/ALM or those with FLT3/WT, suggesting a causal association between FLT3/ITD

and 13qLOH. Further evaluation of copy numbers in those with 13qLOH demonstrated no copy number alterations (no deletion), demonstrating that the observed allelic variation is due to copy neutral LOH, otherwise referred to as acquired segmental uniparental disomy (aUPD). Recent studies have demonstrated the presence of aUPD in not unique to FLT3/ITD and that aUPD may be a common event in myeloid pathogenesis.(61-65) Acquired segmental UPD is a mechanism by which a homozygous state is achieved after an initial acquisition of a heterozygous mutation, where the mutant allele is used as a template, and the wild type allele is converted to that of the mutant one, resulting in a homozygous state. We have demonstrated that in FLT3/ITD specimens with previously demonstrated 13q aUPD, CD34+/CD33- early myeloid progenitors exhibit FLT3/ITD in a heterozygous state (no aUPD), and homozygous state is observed only in the more mature, CD34+/CD33+ myeloid blasts, confirming that aUPD is a late event in the pathogenesis of AML.(60) Segmental UPD may involve a region within the allele resulting from two symmetrical break followed by repair, resulting in conversion of an intestitial region within the chromosome (interstitial segmental UPD, Figure 2C) or from a single break, leading to conversion of the entire telomeric segment of the chromosome (terminal segmental UPD, Figure 2D).

Therapeutic Interventions in Patients with FLT3 Mutations.

Identification of FLT3 mutations in AML has raised the potential for its utility as molecular marker for risk-based therapy and the utility of the FLT3 mutation for targeting with novel small molecular inhibitors. There are now a number of studies available that have examined allogeneic hematopoietic cell transplantation (HCT) and novel small molecular inhibitors in patients harboring FLT3 mutations. We will review the data on the role of HCT as well as the utility of FLT3 inhibitors in the management of patients with high-risk FLT3 mutations.

Role of HCT in FLT3/ITD+ AML.

AML patients with FLT3/ITDs have lower CR rates as those without FLT3/ITD, however, those who achieve a CR remain at significantly higher risk of relapse and death.(24, 27, 28, 66) Post-remission intensification therapies including allogeneic stem cell transplantation has been evaluated for its role in the treatment of AML patients with FLT3/ITDs. A recent study examined whether patients who received allogeneic HCT in initial CR had an improved clinical outcome compared to those who were treated with conventional chemotherapy.(67) Those patients with FLT3/ITDs who received an allogeneic HCT from a matched related donor had a lower RR (22% vs. 49%) than those patients who received standard chemotherapy. More recent adult and pediatric studies confirmed these finding by demonstrating that patients with FLT3/ITD benefited from allogeneic HCT in first CR.(28, 68) Therefore, the overall results suggest that although FLT3/ITDs are predictive of relapse and poor outcome in the chemotherapy setting, its prognostic significance

may be abrogated with allogeneic HCT. This improvement in clinical outcomes with allogeneic transplantation for FLT3/ITD positive AML is very similar to the situation found for Philadelphia positive ALL where disease is associated with dismal outcome with chemotherapy alone, but not so with allogeneic HCT.

Small Molecule Inhibitors as Therapeutic Options

The FLT3 pathway is an obvious target for tyrosine kinase inhibitors (TKIs), as FLT3 mutations are one of the most common mutations in AML and constitutively activate the receptor kinase. Since over-expression of FLT3-WT and expression of FLT3 mutations have been implicated in leukemogenesis, small molecule inhibitors that block the constitutive activation of the mutated and even wild-type receptor may have therapeutic implications.(69) Initial *in vitro* studies using nonspecific TKIs (herbimycin A, AG1296, and AG1295) found that these drugs blocked constitutive activation of FLT3/ITDs and preferentially killed cells harboring FLT3/ITDs.(70-73) Subsequent screens identified numerous other potential compounds (MLN518, PKC412, SU5416, SU5614, SU11248, CEP-701, CEP-5214) that also block FLT3 activation.

Two compounds (CEP-701 and PKC-412) have demonstrated some therapeutic promise for AML patients with FLT3 mutations. CEP-701 (Lestaurtinib) is an indolocarbazole compounds that inhibits autophosphorylation of the WT and mutant FLT3 receptors (74, 75) with high selectivity against FLT3 compared to other RTKs such as KIT, FMS, and PDGF.(74) A phase I/II trial evaluated single agent CEP-701 in patients with refractory, relapsed, or poor risk AML expressing FLT3-activating mutations.(76, 77) Five of fourteen AML patients with FLT3 mutations achieved an objective clinical response with CEP-701 although no CRs were observed.(76, 77) They also demonstrated that CEP-701 is highly plasma protein bound and lack of FLT3 inhibition in some instances may be due to lack of drug availability.(78, 79) In evaluating the in vitro efficacy of CEP-701 in FLT3/ITD, Levis et al. demonstrated that CEP-701 combined with chemotherapy killed cell lines harboring a FLT3/ITDs in a synergistic fashion,(79) suggesting a possible therapeutic benefit to adding CEP-701 with chemotherapy. They subsequently evaluated the efficacy of combining CEP-701 conventional chemotherapy.(78) A total of 48 AML patients with FLT3 mutations were randomized to either receiving standard chemotherapy or standard chemotherapy with CEP-701 during first relapse. Of the 24 patients who received CEP-701, 5 achieved complete CR and another 5 obtained a CR with incomplete count recovery. For those patients receiving only standard chemotherapy, 3 achieved CR and an additional 3 obtained CR with incomplete count recovery. CEP-701 is also being evaluated in a phase III trial for its efficacy in de novo AML with FLT3 mutations in MRC 15, where patients with FLT3 mutations are randomized to standard chemotherapy with or without CEP-701.

PKC412 (Midostaurin) is a benzoylstaurosporine, which was initially developed as a VEGF receptor inhibitor but also inhibits FLT3 receptor kinase.(80) A phase II trial examined PKC412 as a single agent in 20

high risk AML patients with FLT3/ITDs. Although 6 patients demonstrated objective response, again no CRs were observed.(81) The also observed that autophosphorylation of the mutant receptor was blocked in most of the responding patients, indicating an *in vivo* target response using the dose in the study.(81) Subsequently, PKC-412 was combined with daunorubicin and cytarabine in 40 patients with *de novo* AML.(82) The CR rate for those patients with FLT3-WT was 69% compared to >90% for those patients with FLT3 mutations, suggesting possible therapeutic advantage in patients with FLT3/ITDS for combining PKC412 with standard chemotherapy. A recent phase III study is randomizing 514 patients with de novo AML to daunorubicin, cytarabine, with or without PKC-412 in order to define the clinical efficacy of this combination therapy.

These data suggest that FLT3 inhibitors as a single agent has a limited role in the treatment of AML, although their addition to conventional chemotherapy *may* provide therapeutic benefit for patients with FLT3/ITDs and perhaps other molecular alterations. Given the biological and clinical heterogeneity of FLT3 mutations, future studies will need to determine which AML patients with FLT3 mutations have the highest likelihood to respond to these novel drugs in order to appropriately target agents to those patients who are most likely to benefit.

Summary

Since the discovery of FLT3 mutations in 1996, there has been significant interest in examining the molecular biology and clinical significance of these mutations. The current data suggest that FLT3 mutations are not sufficient for leukemic transformation and it requires other cooperating genomic alterations for evolution of leukemic phenotype. In addition, the biology of these mutations is quite complex, with significant heterogeneity of the mutation type (ITD vs. ALM), expression level, size and allelic ratio, which impacts its biology and clinical response. With evolving data on acquisition of LOH in patients with FLT3/ITD, it appears that clonal evolution of cells with FLT3/ITD may lead to homozygous state (aUPD) and impact disease biology and clinical outcome. In fact there is evolving data that aUPD may be a more common event than previously recognized in the evolution of AML. Our expanded knowledge into the genetic variability of FLT3 has emphasized the extent of the complexity in AML. Further understanding of how such genomic complexities impact signal transduction would enable more appropriate targeting of the signal transduction pathway.

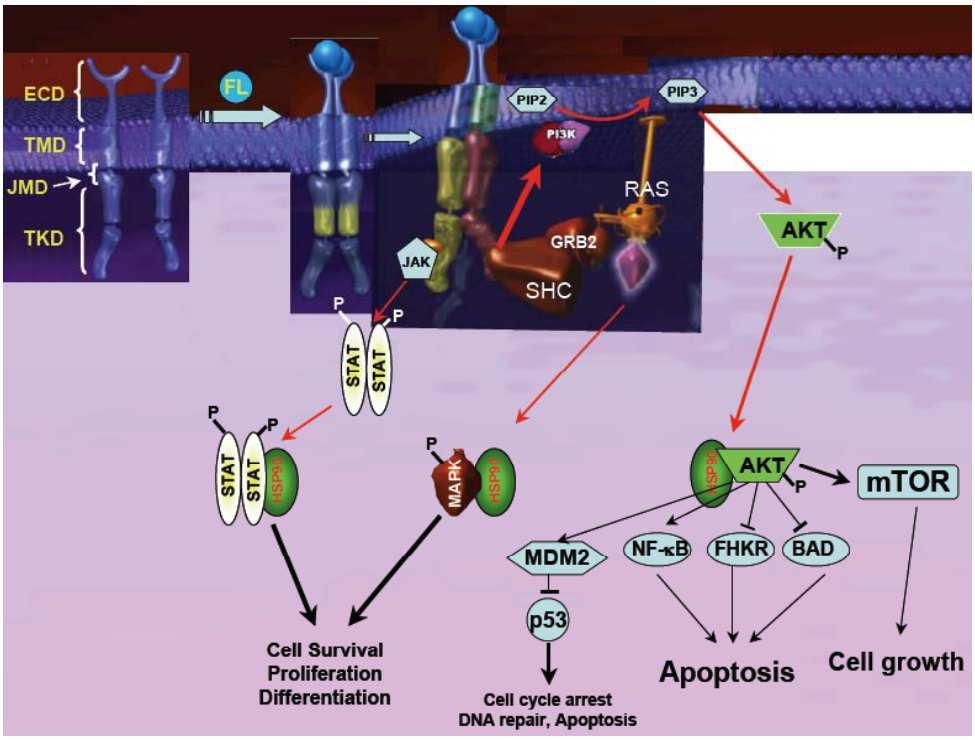

Figure 1. FLT3 signal transduction pathway: FLT3 receptor monomer is composed of an extracellular domain (ECD), a transmemberane domain (TMD), a Juxtamembrane domain (JMD) and a tyrosine kinase domain (TKD) interrupted by a short kinase insert. Binding to FLT3 ligand (FL) leads to receptor dimerization and activation of the intracellular kinase. Tyrosine kinase activation leads to phosphorylation of multiple sites in the intracellular kinase moiety. The activated receptor recruits a number of proteins in the cytoplasm including SHC and GRB2 to form a complex of protein-protein interactions, leading to activation of a number of intracellular mediators including AKT, MAPK and STAT. Activated mediators interact with HSP90 which protects them from inactivation and chaperones the active mediators to the nuclear interphase, where they are released into the nucleus and act to mediate vital cellular functions including cell growth, differentiation, apoptosis, DNA repair and proliferation.

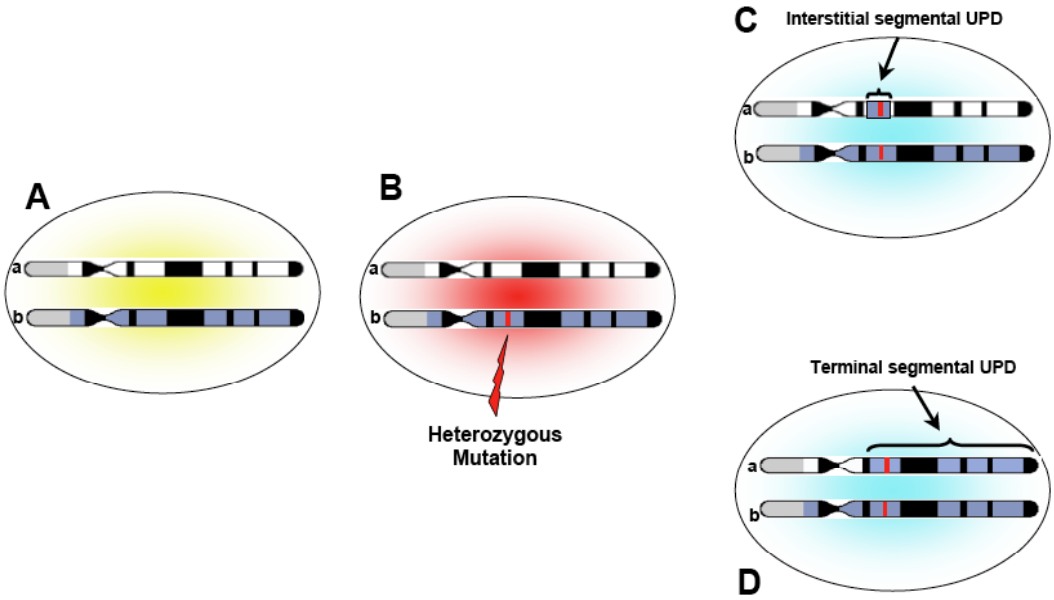

Figure 2. Loss of heterozygosity (LOH) as a due to aUPD. Acquired uniparental disomy, resulting in homozygous state can involve the entire chromosome or be confined to a segment of a chromosome (Segmental aUPD). Segmental aUPD is mediated through homologous recombination mediated somatic crossing over between two homologous non-sister chromatids. In baseline, non mutated state, genes are represented by two different alleles (A, heterozygous state). Molecular alteration is acquired in one allele, leading to a heterozygous mutation (B). Evolution of UPD leads to conversion of the wild type allele to mutant, and evolution of homozygous mutation. When interstitial, the segmental UPD results from two symmetrical breaks flanking a segment of the chromosome, where during repair process, the region flanked by the breaks in the wild type allele is used as a template and as a result, a segment of chromosome undergoes conversion from heterozygous to homozygous state (acquired UPD). Terminal segmental UPD results from a single symmetrical break in each of two homologous non-sister chromatids and the resultant repair product demonstrates UPD (LOH) of the region telomeric to the region of double strand break.

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