

**Short title:** Double mutant *DNMT3A* AML: a unique subtype

**Title:** Double mutant *DNMT3A* AML: a unique subtype experiencing increased DNA damage and poor prognosis

**Introduction:** Mutation of *DNMT3A*, encoding a *de novo* methyltransferase essential for cytosine methylation, is a common early event in clonal hematopoiesis (CH) and adult acute myeloid leukemia (AML). Spontaneous deamination of methylated cytosines damages DNA, which is repaired by the base excision repair (BER) enzymes MBD4 and TDG. Congenital MBD4-deficiency has been linked to early-onset CH and AML, and is marked by excessive levels of DNA damage and mutation of *DNMT3A*. Strikingly, wildtype (WT) *DNMT3A* binds TDG, thereby potentiating its repair activity. Since TDG is the only remaining BER enzyme in MBD4-deficient AML patients, we investigated whether mutant *DNMT3A* negatively affects the capacity of TDG to repair methylation-linked DNA damage.

**Methods:** MBD4 and TDG repair activity was assessed *in vitro* in the presence of varying concentrations of recombinant wildtype or mutant *DNMT3A* (R635W, R688C, R882C, A884V) using glycosylase assays. Differences in survival and co-mutation was assessed in our AML cohort comprising 1.872 *DNMT3A* WT, 585 SM and 88 DM patients. Whole-genome sequencing (WGS) was performed on bone marrow aspirates from 22 AML patients, comprising 6 *DNMT3A* WT, 7 SM and 9 DM patients to assess whether methylation-linked DNA damage increases dependent on the *DNMT3A* mutation type or number. AlphaFold 2.0 was used to model the interaction between *DNMT3A* and TDG to determine the impact of *DNMT3A* mutations positioned at the interaction interface.

**Results:** We found that, whereas WT *DNMT3A* stimulates TDG function, mutant *DNMT3A* impairs TDG-mediated repair of DNA damage *in vitro*. In light of this finding and to extrapolate our observations to the broader AML patient population, we investigated the genetic profiles and survival outcomes of AML patients with single (SM) versus double mutant (DM) *DNMT3A*. DM *DNMT3A* AML patients show a characteristic driver mutation landscape and reduced overall survival when compared to SM *DNMT3A* AML patients. Importantly, whole-genome sequencing showed a trend for increased DNA damage in primary DM *DNMT3A* AML samples, especially when *DNMT3A* mutations are located at the *DNMT3A*-TDG interaction interface.

**Conclusion:** We show that biallelic mutant *DNMT3A*, a subgroup comprising approximately 4% of the complete AML population, is linked to increased methylation-linked DNA damage, diverging driver landscape and unfavorable overall survival.