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Development of an isothermal method for the detection of DNA hypermethylation

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RIASSUNTO

Il presente lavoro di tesi tratta dello sviluppo di un nuovo metodo isotermo per la rilevazione degli stati di ipermetilazione del DNA, la Methylation-Specific Loop-mediated Isothermal Amplification (MS-LAMP).
La metilazione del DNA è il principale meccanismo epigenetico riscontrabile negli organismi multicellulari. Il processo di metilazione è catalizzato da enzimi metil-transferasici che promuovono il legame covalente di un gruppo metile all'anello pirimidinico di una citosina immediatamente antecedente una guanina. I dinucleotidi CpG possono trovarsi raggruppati, all'interno del genoma umano, in veri e propri clusters, le cosiddette "CpG islands" che caratterizzano, ad esempio, la sequenza dei promotori di molti geni del nostro genoma. La metilazione ha effetto sull'interazione tra il DNA e alcune proteine e, generalmente, uno stato di ipermetilazione delle CpG islands all'interno di promotori ha la funzione di silenziare l'espressione della relativa proteina, impedendo il legame di specifici fattori di trascrizione o dell'intero macchinario trascrizionale.
Molte malattie umane sembrano essere correlate con una anomala metilazione del genoma: tra queste si riscontrano malattie autoimmuni, neuro-degenerative e soprattutto il cancro.
L'origine del fenotipo degenerato di una cellula cancerosa può dipendere originariamente da molti fattori quali mutazioni della sequenza di DNA o alterazioni macroscopiche del genoma ma anche la metilazione del DNA può giocare un ruolo in questo contesto, principalmente inibendo il legame di fattori di trascrizione ai promotori di geni oncosoppressori o, viceversa, attivando promotori di protooncogeni, retrotrasposoni o geni correlati con instabilità genomica o metastasi.
La rilevazione di una metilazione aberrante del DNA può quindi essere applicata alla diagnosi tumorale in diversi modi, tra cui l'individuazione diretta del cancro in prelievi bioptici, la predizione del comportamento della malattia se lo stato di metilazione è indicativo di una determinata caratteristica patologica o l'individuazione precoce del fenotipo tumorale in tessuti non ancora cancerosi.
MS-LAMP è quindi proposta come una nuova metodica isoterma real-time per la rilevazione degli stati di ipermetilazione del DNA. Come per la maggior parte delle tecniche utilizzate ad oggi per la rilevazione della metilazione delle CpG islands, anche MS-LAMP prevede che il DNA sia preventivamente trattato chimicamente mediante bisolfito di sodio che è in grado di convertire tutte le citosine del DNA da analizzare, in uracili, con l'eccezione delle citosine metilate dei dinucleotidi CpG che invece rimangono protette. Questo processo converte quindi una modificazione

chimica (metilazione) stabile ma non rilevabile con i metodi della diagnostica molecolare in una differenza di sequenza tra fenotipo metilato e non metilato e quindi rilevabile con metodiche quali PCR, digestione enzimatica o MS-LAMP.
Negli ultimi anni sono state sviluppate diverse tecniche di amplificazione del DNA alternative alla PCR, con prestazioni paragonabili e caratteristiche peculiari che le rendono potenzialmente vantaggiose per applicazioni diagnostiche. Una di queste è la "Loop-mediated Isothermal Amplification" (LAMP).
LAMP è una metodica isoterma di amplificazione del DNA alternativa alla PCR, sviluppata recentemente e che prevede l'utilizzo di una polimerasi termostabile dotata di attività di "strand displacement" e di un set di 6 primer espressamente disegnati per riconoscere zone distinte del DNA target. Mediante una specifica dinamica di *annealing* e *displacement* che conduce alla formazione di loops, è possibile amplificare il materiale genico di partenza fino a 10^{10} volte mantenendo la temperatura costante intorno all'optimum di attività della polimerasi, in tempi brevi (meno di 60 minuti) e con elevatissima specificità. L'esito della reazione è valutabile sia in fluorescenza, mediante metodiche tradizionali come intercalanti o *molecular beacons* o meno diffuse, quali il quenching da guanine, sia mediante turbidimetria. Questo metodo di rilevazione è reso possibile dell'elevata efficienza di amplificazione propria della LAMP e consiste nel misurare l'intorbidimento della miscela di reazione che è riconducibile alla precipitazione di pirofosfato di magnesio. Questo sale insolubile si forma dall'interazione degli ioni Mg^{2+} presenti in soluzione con il pirofosfato inorganico liberato dai dNTPs in seguito all'incorporazione dei nucleotidi nei filamenti nascenti di DNA. La quantità di pirofosfato di magnesio è quindi proporzionale alla quantità di DNA prodotto ed è misurabile come segnale di trasmittanza, sia a fine reazione per una valutazione qualitativa che in tempo reale per un approccio quantitativo. Per la sua semplicità e praticità la turbidimetria rappresenta quindi la tecnica di rilevazione d'elezione per LAMP.
La LAMP rappresenta quindi un interessante campo di ricerca e sviluppo per la progettazione di nuovi saggi diagnostici grazie alle sue caratteristiche di rapidità, specificità, basso costo dei reagenti e della strumentazione richiesta (non sono infatti necessarie termociclazione e fluorescenza).
A partire da un'analisi di letteratura sono stati quindi scelti come sequenze target dei nostri saggi 3 promotori del genoma umano il cui stato di ipermetilazione in campioni clinici è stato correlato con l'insorgenza di tumori al polmone: quello per il gene della "Death-assiociated protein kinase" (DAPK), del "Cyclin-dependent kinase

inhibitor 2A" (CDKN2A o p16) e della "GATA binding protein 5" (GATA5).
I set di primers sono stati quindi disegnati in modo da contenere un certo numero di dinucleotidi CpG che permettessero un'efficiente discriminazione tra i due stati di metilazione, amplificando il solo DNA metilato. I primers sono stati disegnati in modo da non contenere punti di discriminazione (CpGs) in corrispondenza delle zone estensibili dei primers, normalmente le più efficienti per la discriminazione di target mutato; questo razionale è stato adottato con il fine di limitare, in caso di campioni clinici parzialmente metilati, il rischio di falsi negativi dovuti al mancato annealing di uno o più primers al relativo target. La specificità è mantenuta, in LAMP, grazie alla simultanea attività di 8 regioni di appaiamento, la cui necessaria operatività garantisce l'amplificazione del solo target previsto.
Le reazioni sono state condotte in un blocco termico appositamente progettato per LAMP e dotato di un rilevatore in Real-Time di torbidità o in un termociclatore (usato in condizioni isoterme) dotato di rilevatori di fluorescenza per alcune applicazioni in fluorescenza.
La valutazione delle prestazioni della tecnica è stata effettuata su dei target modello e suddivisa in due parti sulla base del tipo di target utilizzato.
In un primo momento il set di primers relativo al promotore DAPK è stato stati testato su mini-geni sintetici, come prova di principio. Questi plasmidi contengono già all'origine la sequenza nei due stati (metilato o non metilato) in seguito al trattamento con bisolfito. In questo modo sono state quindi valutate le prestazioni dei saggi inizialmente senza dover effettuare il trattamento chimico, possibile fonte di variabilità. Sono state dunque testate la specificità (capacità di amplificare il target specifico a dispetto di quello non specifico), la sensibilità (minima concentrazione di target metilato rilevabile) e selettività (minima concentrazione di target metilato rilevabile in presenza di target non metilato). Questi esperimenti hanno confermato la capacità dei primer disegnati di distinguere tra DNA metilato e non metilato sulla base della sequenza derivante dal trattamento con bisolfito, con prestazioni in termini di sensibilità e selettività soddisfacenti.
Si è successivamente passati alla valutazione delle prestazioni dei 3 saggi mediante l'utilizzo di DNA genomici di controllo come target modello per le reazioni: si tratta di DNA genomici umani commerciali interamente metilati o non metilati (su tutti i dinucleotidi CpG) che necessitano di trattamento con bisolfito di sodio prima di essere saggiati e rappresentano il formato di target più vicino al vero campione clinico. Anche in questo caso è stata testata la specificità, che si è dimostrata ottima (rilevazione del solo target metilato, per tutti e tre i sistemi nei 60 minuti di reazione) e la selettività che ha permesso di concludere che la

tecnica è in grado di rilevare DNA metilato in quantità mediamente comprese tra 1% e lo 0.25% rispetto a un background non metilato.
Un'ulteriore fase del progetto ha previsto la valutazione della fattibilità di un saggio multiplex che prevede la contemporanea presenza dei 3 saggi in un'unica reazione, consentendo un risparmio in termini di tempo e reagenti. Nelle tradizionali tecniche di diagnostica molecolare la riuscita di questo tipo di analisi è normalmente resa non triviale soprattutto dalla propensione con cui i primers interagiscono tra di loro, generando prodotti di amplificazione aspecifici (dimeri di primer). Il meccanismo di amplificazione di LAMP e in particolare l'assenza di una denaturazione termica ciclica, rendono di fatto molto improbabile la propagazione di dimeri aspecifici e permette di unire in una sola reazione almeno 3 saggi distinti senza che i 18 primers che complessivamente li compongono interagiscano in maniera da produrre risultati falsi positivi. L'applicazione multiplex è stata esplorata con buoni risultati mediante due approcci distinti di rilevazione del segnale: il primo è stato quello tradizionale e più tradizionalmente associato a LAMP, cioè la turbidimetria. Per quanto questa tecnica di rilevazione del segnale non consenta di distinguere l'amplificazione dei singoli geni, questo approccio rimane utile in campo clinico; permetterebbe infatti un rapido screening iniziale dei campioni alla ricerca di un generico segnale di ipermetilazione, prima di una più approfondita analisi dei singoli promotori senza richiedere strumentazione complessa e costosa e comunque in tempi molto ridotti.
È stata successivamente anche valutata la fattibilità di una reazione LAMP in multiplex dove la rilevazione del segnale fosse effettuata mediante la fluorescenza. L'uso simultaneo di differenti fluorofori che emettono luce fluorescente a diverse lunghezze d'onda consente di distinguere l'amplificazione di ciascuno dei tre distinti saggi indipendentemente. La modalità di rilevazione in fluorescenza dello svolgimento della reazione è stato il quenching da guanine: un fluoroforo che, legato ad un oligonucleotide che ibridizza su un DNA target, si trova in prossimità di una o più guanine smorza in maniera netta la sua fluorescenza nativa e il segnale è rilevabile in tempo reale in un termociclatore per real-time PCR utilizzato in condizioni isoterme. Ognuno dei tre differenti fluorofori è stato quindi legato ad uno dei due loop primers di uno dei saggi e la specificitá della reazione in triplex é stata valutata e confermata su differenti miscele dei diversi target modello.
Alla luce dei risultati molto incoraggianti ottenuti testando i nuovi saggi LAMP sui modelli, si è proceduto alla valutazione della funzionalità della tecnica su un limitato numero di campioni clinici. A tal fine sono stati reperiti campioni di resezioni tumorali di adenocarcinoma polmonare forniti da una collaborazione con il Dana-Farber Cancer

Institute di Boston (MA, USA) che hanno consentito di testare le prestazioni di questa nuova tecnica in un vero contesto clinico, confrontandola con un metodo riconosciuto. I test hanno mostrato che la tecnica funziona anche su campioni clinici con buoni risultati pur evidenziando alcuni dati discordanti che aprono la strada, a partire dai dati incoraggianti già ottenuti, a considerazioni e a spunti nuovi per l'applicazione di LAMP alla rilevazione degli stati di ipermetilazione del DNA.

INTRODUCTION

Epigenetics

Epigenetics, according to one of the first definitions conceived in 1942 by Conrad Waddington, can be considered as the "interaction of the genes with their environment that bring the phenotype into being". Moreover, nowadays, we can refer to epigenetics as a branch of biology or molecular biology that studies the mechanisms by which gene expression states, somatically heritable, are controlled without changes in the underlying DNA sequence. Epigenetic mechanisms in mammal organisms include DNA methylation, modification of the post-translational state of histones, such as by methylation and acetylation, chromatin modification by proteins like Polycomb and Trithorax and the increasingly important control of mRNA expression by small non-coding RNAs[1] (**Figure 1)**.

If less is known about epigenetic mechanisms comparing to genetic ones, it is though possible to depict the differences between the two control systems: genetics is based on cell lineages and clonal inheritance and if a mutation or a bigger chromosomal change occurs, this will be expected to appear in all the descendants.

Epigenetic modifications, instead, normally occur in limited group of cells, such as, for instance, in the developing tissues from common cellular progenitors even if also some clonal events like X-chromosome inactivation, are driven by epigenetic mechanisms. Moreover the epigenetic modifications can be reverted while the mutations in the DNA sequence persist throughout generations. Finally, the environment, which is able to put pressure and select phenotypic characters, is not capable of interfering with genetic ones; oppositely, epigenetics can be often regulated by environmental contributions, inducing changes which are then heritable and which, in some way, escape from Darwinian theories, encompassing a Lamarckian model[2,3].

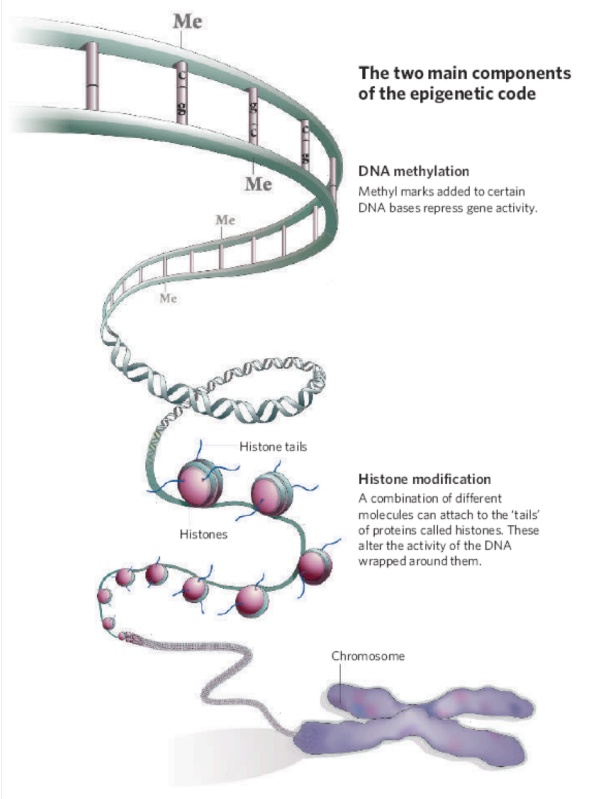


Fig. 1 DNA organisation and epigenetic modifications.

Chromatin modification

Chromatin is the molecular structure that packages the DNA macromolecule in an ordered and regulated system. The basic building block of chromatin is the nucleosome, consisting of 146 bp of DNA wrapped around an octamer protein core consisting of two subunits of each histone, H2A, H2B, H3, and H4. In between core nucleosomes, the linker histone H1 attaches and facilitates further compaction in order to arrange nucleosomes into higher order structures[4].
Regulation of chromatin structure is a complex and dynamic process, regulated at different levels by several molecular mechanisms such as DNA methylation, nucleosome remodeling, histones modifications and non-coding RNA.

Histones are recognized as being translators between genotypes and phenotypes, with a dynamic function in the regulation of the chromatin structure and the gene activity, as a compact condensation status of the chromatin corresponds to a gene silencing while, on contrary, an open unpackaged structure leads to the expression of the genes of a peculiar genomic area. Histones can be the target of a great number of post-translational modifications: acetylation, methylation, phosphorylation, sumoylation, ubiquitination, deimination, ADP-rybosilation and proline cis-trans isomerisation[5]. Among these, acetylation of lysine residues of certain N-terminal tails by HAT enzymatic activities (Histone Acetyl Transferase) is directly linked to an active trascription while the reverse modification, catalyzed by histone deacetylases (HDAC) suppresses the DNA expression[6,7,8]. Histone methylation is also an important regulation factor for chromatin status but it is correlated with different functional states of chromatin depending on which residues are modified and by which extent: tri-methylation of lysine 4 of H3 and mono-methylation of lysines 9 and 27 of H3 seem to prelude to gene activation while di- and tri-methylation of lysine 9 and 27 leads to gene repression[9,10,11].

miRNA

miRNAs represent one of the most recently discovered participants in the epigenetic fields. MicroRNAs are noncoding RNAs with key regulatory functions and involved in many biological processes spanning from development, differentiation, and cell cycle regulation to senescence and metabolism[12]. MiRNA genes are transcribed in the nucleus by an RNA polymerase II. The first transcript is a long, capped, and polyadenylated precursor (pre-miRNA) which is subsequently processed by a ribonuclease III (called Drosha), in conjunction with its binding partner DGCR8 (Pasha), into a 70-100-nt hairpin-shaped RNA called pre-miRNA. Translocated to the cytoplasm, pre-miRNA is cleaved into a 18-24-nt miRNA duplex by a ribonucleic complex composed of a ribonuclease III (Dicer) and TRBP. The duplex binds to a large protein complex called RISC (RNA-induced silencing complex). One strand of the miRNA duplex remains stably associated with RISC and drives the other strand (the proper mature miRNA) to its target mRNAs, which can undergo cleavage (perfect miRNA:mRNA complementarity) or translational repression (imperfect complementarity). The full spectrum of miRNAs expressed in a specific cell type (the miRNome) varies between normal and pathologic tissues and specific signatures of deregulated miRNAs harbor diagnostic and prognostic implications[13].

DNA methylation

DNA methylation represents the major epigenetic control mechanism in multicellular organisms; in mammalian cells it is mainly observed on cytosine residues within CpG dinucleotides.
DNA methylation is regulated by a family of DNA methyl transferases (DNMTs) which transfer a methyl group from the methyl donor S-adenosylmethionine (SAM) to the carbon-5 position of cytosine (**Figure 2**). Human DNMTs family include many different enzymes among which the most relevant are DNMT1, DNMT3A and DNMT3B[14]. Methylation can be either a "de novo" process if cytosines on both strands of CpG dinucleotides result unmethylated or a maintenance process when CpG is methylated on one of the two DNA strands. DNMT1 shows both the activities while DNMT3A and DNMT3B act mainly as de novo methyltransferase[15]. Thus DNMT3A and 3B are important for determining the correct pattern of DNA methylation during embryogenesis, which is then kept by DNMT1 acting as maintenance methyltransferase.

In normal cells the function of DNA methylation is diverse and it includes several processes such as silencing of transposable elements, inactivation of viral sequences, maintenance of chromosomal integrity, X chromosome inactivation, and gene expression[16,17,18,19,20,21,22].
CpG dinucleotides are underrepresented in the genome, ranging between 2 and 5% of the entire genome. Their distribution is also not uniform throughout the genome: 80% of the CpG dinucleotides are mainly localized within repetitive elements, satellite sequences, rDNA and centromeric regions. These CpG dinucleotidides are usually heavily methylated in order to keep these areas silenced.
The remaining 20% of CpGs are gathered in short stretches of DNA where the CpGs content is very high, also called "CpG islands", which are located in the promoter regions of approximately half of the human genes: the majority of these genes have housekeeping functions and in this case the CpGs are usually found in the unmethylated state to allow the translation of the DNA[23].
DNA methylation determines the state of gene expression either autonomously or interacting with histone-modification mechanisms: DNMTs recruit HDACs, leading to histone deacetylation and transcriptional repression[24]. Further, methylated DNA is recognized by methylated DNA binding proteins (MBPs), which also recruit HDACs and ATP-dependent chromatin-remodeling proteins, resulting in chromatin condensation and gene inactivation.

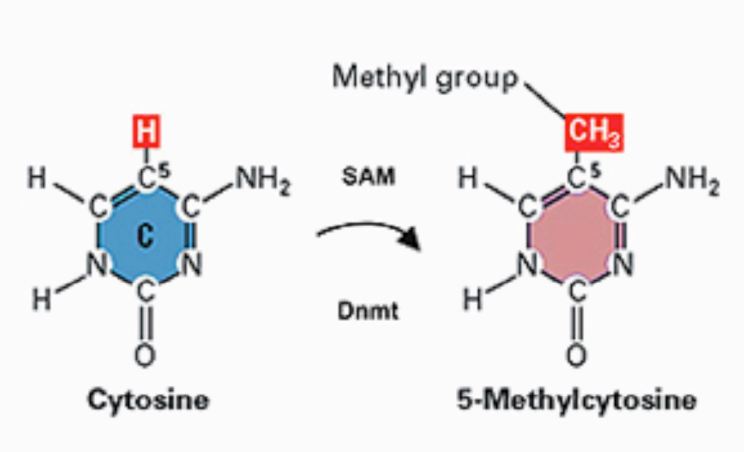


Fig. 2 Reaction of methylation: methyl-group transfer from S-adenosyl methionine onto C5 of cytosine is catalyzed by a DNA methyl transferase

CpG island promoter methylation can additionally block transcription by interfering with binding of transcription factors and translational machinery to their target binding sites[25,26] (**Figure 3**).

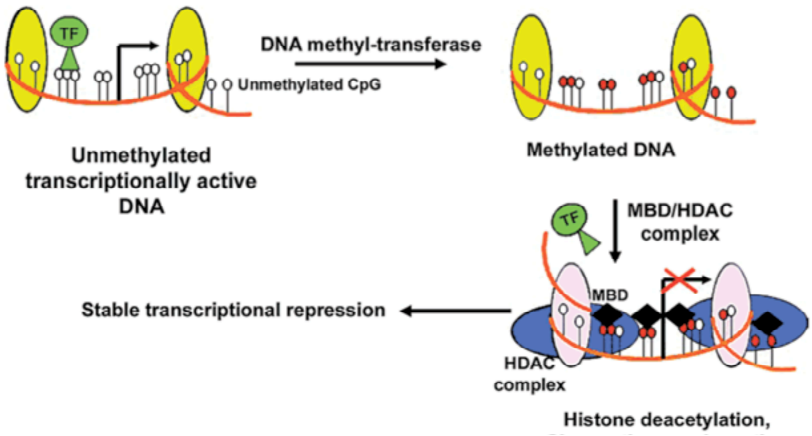


Fig. 3 Mechanism of gene silencing mediated by DNA methylation and histones modification

The link between histones modification, DNA methylation and nucleosomal remodeling has been recently explored. Nucleosome/chromatin remodeling complexes proteins NuRD and Brahma were shown to interact respectively with MBD2 and MeCP2, members of the families of the methylated DNA binding proteins[27,28]. These experiments provide a potential link between DNA methylation and chromatin silencing and other studies suggest that these processes are linked to chromatin remodeling by specific ATP-dependent remodeling machines[29,30]. These data suggest an intimate link between DNA methylation, histone modification and nucleosomal remodeling (**Figure 4**) and that alterations in this balance could lead to permanent silencing of important genes, related to many diseases.

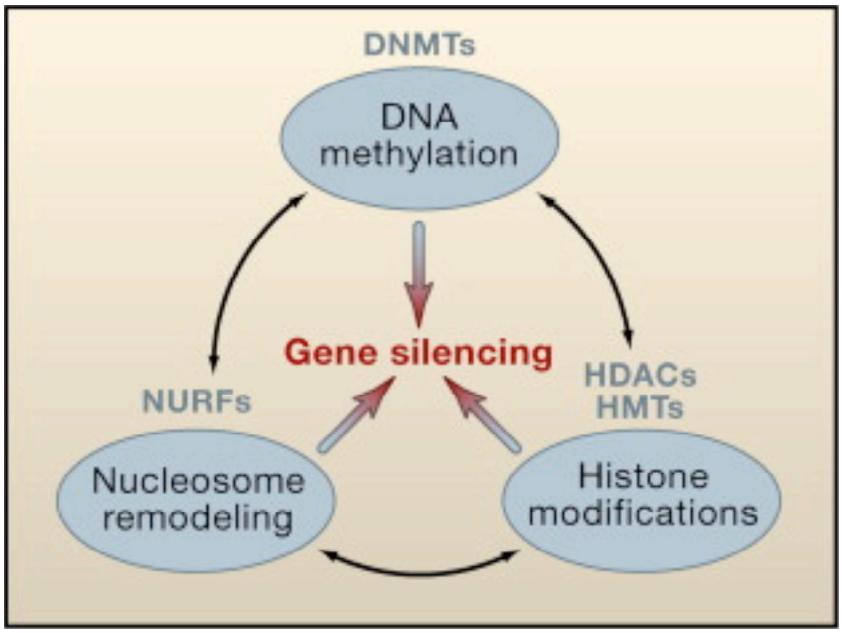


Fig. 4 Interaction between main epigenetic mechanisms for gene translation silencing

DNA methylation-related diseases

DNA methylation is one of the most important mechanisms, not involving changes in DNA sequence, for the control of the translation of a relevant amount of human genes. An aberrant methylation can lead to serious diseases due to the transcriptional state of promoters of key human genes either in case of hypomethylation of promoters normally methylated (de-silencing) and in case of hypermethylation of promoters normally unmethylated (silencing).
DNA methylation levels and profile are very dynamic, especially during the determination of the epigenetic pattern of the organism which takes place very early during the embryogenesis. In a first phase the partly methylated genomes derived from the two gametes undergo a global demethylation process. Methylation is then re-established, starting in the immediate post-conception period. Imprinted genes seem to keep the methylation profile which was specific of the parental origin[31,32] (**Figure 5**).

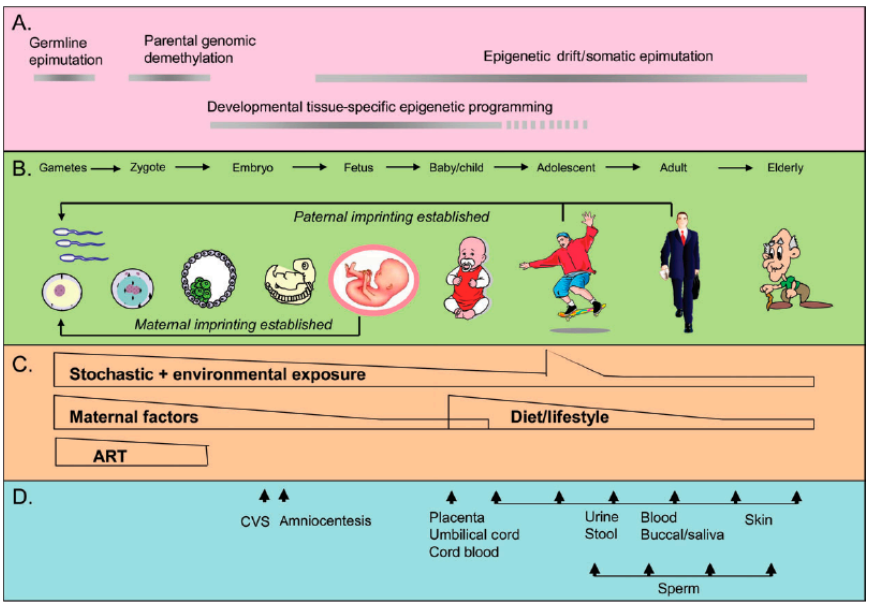


Fig. 5 Epigenetics, environment and developement

Aberrations in the methylation pattern acquired during embryogenesis can lead to congenital disorders and to several different diseases involving many key regulations of human organisms

Imprinting diseases

Imprinting patterns have been associated with congenital disorders affecting growth and neurodevelopment that persist into adulthood, including Prader-Willi and Angelman syndromes, which are two clinically distinct diseases associated with abnormal imprinting on chromosome 15q11-q13.
Loss of maternal imprinting is responsible for the Angelman syndrome, which is characterized by mental retardation, ataxia, and social disposition.
In Prader-Willi syndrome, loss of paternal imprinting in the same region is characterized by learning difficulties, hypogonadism, short stature, and small hands and feet[33]. Beckwith-Weidemann syndrome, another imprinting disorder characterized by macrosomia, hemihypertrophy, abdominal wall defects, organomegaly, and susceptibility to Wilm's tumor, is the result of loss of imprinting of insulin-like growth factor 2 (IGF2) on chromosome 11p15[34].

Autoimmune diseases

DNA methylation plays an important role in maintaining T-cells function. Failure in the regulation of patterns and levels of the DNA methylation in mature T-cells results in autoreactivity *in vitro* and autoimmunity *in vivo*.
Systemic Lupus Erithematosus (SLE) is a serious autoimmune disease where an aberrant DNA methylation profile seems to play an important role: in particular a hypomethylation profile was detected in sera from SLE patients. Moreover a de-methylating agent such as 5-aza C was found to generate SLE like symptoms[35].
Another congenital disorder is the ICF syndrome, an extremely rare autosomal recessive disease that is characterized by profound immunodeficiency, centromeric instability and facial anomalies and is caused by a mutation of the *DNMT3b* gene, leading to defective methylation.

Neural diseases

Expansion and methylation of CGG trinucleotide repeats in the FMR1 gene (fragile X mental retardation) causes fragile X syndrome which is a mental retardation that can occur in 1/4000 male individuals. The molecular basis of this disease is the uncontrolled expansion of CGG repetitions up to 200 times at the untranslated region of FMR1 gene and their hypermethylation, which leads to the silencing of the gene[36]. Other fragile sites on the genome show similar structures as FMR1 gene and suggest a common fate for repeated DNA as an easy target for methylation machinery and silencing.
Amyloid beta precursor protein (APP) is a hallmark of the pathogenesis of Alzheimer disease. Its overproduction and accumulation seems to be related to the loss of epigenetic control on the expression of the protein and lower methylation levels were consistently found in older people[37].

Aging

Evidences for an age-dependent change in DNA methylation are quite extensive. In general, total genomic deoxymethylcytosine (dMC) levels decrease with aging in most vertebrate tissues. Demethylation has been documented in aging salmons, mice, rats, cows and humans and occurs in the brain, liver, small intestine mucosa, heart, spleen and T lymphocytes[38,39] and this status of hypomethylation may promote chromosomal instability, and rearrangements, which increases the risk of over-expression of proto-oncogenes and neoplasia[40].
Methylation changes also occur in some CpG islands and they affect the expression of the associated gene. The CpG island contained in the promoter of the human estrogen receptor has been observed to be hypermethylated with aging in colonic mucosa[41]. A similar age-dependent methylation of the CpG island is associated with the insulin-like growth factor 2 (IGF2) gene, which also displayed a decrease in expression[42].
Age-dependent changes in T cell DNA methylation may contribute to the development of some forms of autoimmunity in the elderly. With aging, T lymphocytes can also develop changes in function and gene expression which include an increase in the memory subset, decreased interleukin-2 (IL-2) production and decreased responsiveness to antigenic stimuli, termed immune senescence[43].

Diet

Diet is an important modifier of epigenetic profile (**Figure 6**). Specific micronutrients involved in one-carbon metabolism include folate, an important primary methyl donor, whose availability is directly correlated with DNA methylation levels[44,45]. Folate, in the form of 5-methyltetrahydrofolate, is a co-factor required for the remethylation of homocysteine to methionine, a precursor of SAM. Since mammals cannot synthesize methyl donor precursors (folate, choline or methionine), dietary ingestion is essential. Low folate levels lead to hyperhomocysteinemia, which inhibits key metabolites of the one-carbon pathway and is associated with coronary heart disease and cancer[46,47].

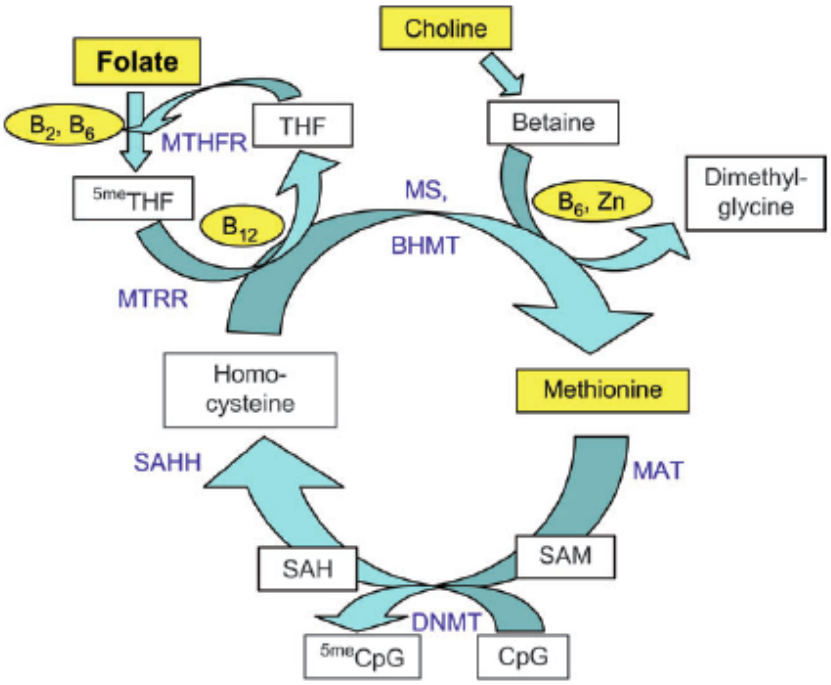
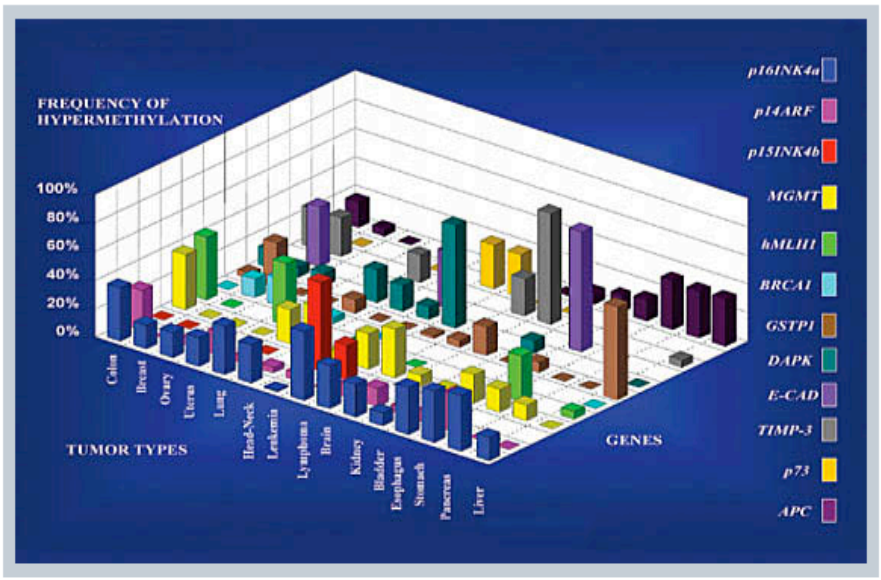


Fig. 6 One-carbon metabolism, which involves the transfer of a methyl group from one ‘‘donor’’ to the next, ending with the DNA methyltransferase (DNMT)–catalyzed transfer of a methyl group to
DNA. SAH, S-adenosylhomocysteine; SAM, S-adenosylmethionine; THF, tetrahydrofolate; 5meTHF, 5-methyl-THF. BHMT, betaine-homocysteine S-methyltransferase; B2, vitamin B2; B6, vitamin B6; B12, vitamin B12; MAT, methionine adenosyltransferase; MS, methionine synthase; MTHFR, 5,10-methylenetetrahydrofolate reductase; MTRR, 5-methyltetrahydrofolate-homocysteine methyltransferase reductase; SAHH, S-adenosylhomocysteine hydrolase; Zn, zinc.

Cancer

It is abundantly clear that mutations, whether inherited through the germ line or, more commonly, arising in somatic tissues later in life, can cause cancer. These mutations abnormally enhance the function of some genes, the oncogenes, or cause other genes, the tumor-suppressor genes, to lose function.
Researchers have argued for decades about whether the initiation and progression of cancer are due only to mutations or, as well, to epigenetic changes that are not caused by alterations in the primary nucleotide sequence of DNA. Not only both views are likely and correct, but also the two processes are intricately connected in driving the development of tumors from the earliest to the latest stages[23]. During cancer development, two distinct changes in DNA methylation occur: genome-wide hypomethylation and locus-specific hypermethylation in promoter-associated CpG islands[48,49]. Both DNA hypo- and hyper-methylation may promote cancer development. Most types of cancer present loss of methylation in the regions where most CpG dinucleotides should be methylated, causing functional instability of chromosomes, loss of imprinting and enhancement of expression of proto-oncogenes (such as cMYC and H-RAS[50]) and inserted viral sequences[51]. In some cases like breast, cervical and brain cancers, a progressive increase in the hypomethylation relates with a progressive grade of malignancy. Gain in methylation of CpG islands in promoter regions of genes normally recognized as tumor-suppressors is the other well established epigenetic de-regulation phenomenon for cancer onset, causing repression of their transcription and tumorigenesis. Numerous genes have been found to undergo hypermethylation in certain cancers and at certain frequencies: these susceptible genes can be involved in cell cycle regulation ($p14^{ARF}$, $p15^{INK4A}$, $p16^{INK4a}$, *Rb*), DNA repair (*BRCA1*, *MGMT*, *hMLH1*), apoptosis (*DAPK*, *TMS1*), drug resistance (*MGMT*), detoxification (*GSTP1*), differentiation, angiogenesis, metastasis (E-cadherin), and signal transduction (*RARβ*, *APC*) (**Figure 7**).

Recent studies seem to show that epigenetic abnormalities might play a role in the earliest steps of cancer onset. Thus gene silencing or abnormal imprinting should constitute a break of a so-called "epigenetic gatekeeper" allowing for subsequent genetic and epigenetic changes which would lead to cancer development[52].
Thus epigenetic silencing of genes as p16, SFRPs, GATA-4, GATA-5 and APC could, in a colon cancer model, promote an abnormal lock of precursor cells in a status of pre-invasive cancer stem cells, which can then be followed by loss of genetic gatekeepers (as APC and b-catenin), finally leading to the formation of the tumor[53] (**Figure 8**).



Source: Esteller et al., Cancer Res, 61:3225-9, 2001

Fig. 7 Frequency of hypermethylated gene in different human cancers.

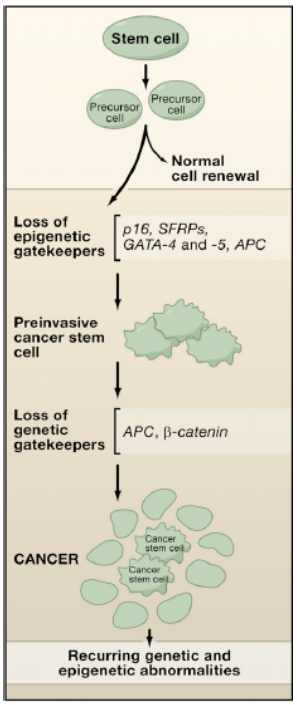


Fig. 8 Epigenetic gatekeepers prevent early tumor progression

Epigenetic loss of function of genes involved in the formation of cancer can be distinguished at some extent from genetically mediated loss of function. Different gene mutations are seldom present at the same time in cancer cells because this can often lead to whole pathway disruption[54]. Moreover, for the same reason, it is also quite infrequent that gene silencing and mutation occur in the same moment. However many multiple epigenetic changes, unlike for mutations, can affect and be detected in a single cell, involving one or more different pathways and also possibly generating more epigenetic changes[55].

Clinical implications of DNA methylation

The major features concerning epigenetic changes consist in their stability, ease of access and detection and reversibility: this can either allow new therapeutic strategies for clinical management of cancer aimed to reverting gene silencing to prevent and/or treat neoplastic diseases and the use of these molecular changes as markers for risk assessment, diagnosis and prognosis.

Therapy

Due to the reversibility of DNA methylation, reactivation of epigenetically silenced genes involved in tumor onset might revert the neoplastic phenotype caused by their repression. The use of demethylating agents such as 5-azacytidine causes the re-expression of silenced genes and can be exploited as a therapeutic opportunity and for cancer prevention, since methylation changes are very often involved in the early stages of tumor formation[56].
5-azacytidine is a deoxycytidine derivative with a modification at the fifth position of the pyrimidine ring and has dual effects on target cells, including the reactivation of silenced genes at low dosage and cytotoxicity at high dosage. The action is irreversible because it involves the formation of a covalent bond between the DNMT and the nucleoside analogue-substituted DNA. Similarly to 5-aza-20-deoxycytidine (decitabine), and zebularine [1-(beta-D-ribofuranosyl)-1,2-dihydropyrimidin-Z-one], that are nucleoside analogues with similar action, 5-azacytidine is converted to the deoxynucleotide triphosphates then incorporated into replicating DNA in place of cytosine. DNA methyltransferases are trapped on DNA containing the modified bases when they try to methylate the modified 5-position, thereby depleting the enzyme and resulting in the formation of heritably demethylated DNA[57].

While being an efficient inhibitor of DNA methylation 5-azacytidine is also toxic to the cells, due to the covalent trapping of DNA-methyltrasferase[58] and due to the fact that, being a ribose nucleoside, it can also be incorporated in the RNA, affecting its function in the cells[59]. Because of these reasons a number of derivatives were also developed as anti-tumor agents: 5'-aza-2'-deoxycytidine, a deoxyribose analogue, is less toxic and more specific for DNA methylation, not being incorporated in the RNA[60]. A newer orally active compound is zebularine; it is significantly less cytotoxic *in vitro* than azacitidine and decitabine but requires higher doses for equivalent inhibition of DNMT[61].
Computational screening approaches where then exploited for the discovery of small molecules able to inhibit DNA methyltransferase but exhibiting less toxic effects not being nuclesides derivatives. Some indene-based heterocycles linked by a 3-carbon bond, possibly binding the enzyme active site, were found to significantly inhibit cytosine methylation in leukemia cells while having much less toxic effects than 5-azacytidine[62].

DNA methylation as molecular marker for cancer

Methylated genomic DNA has several properties that make it an attractive potential biomarker in oncology. Hypermethylation of many genes is negligible in the majority of healthy individuals, even if some exceptions occurs for certain genes whose state of methylation changes due to aging and environmental stresses. Thus the majority of the changes detected in the cells are acquired uniquely during the neoplastic transformation and are specific of cancerous tissues. Profiles or panels of marker gene promoters can be designed, in which the examination of a few gene-hypermethylation markers will be positive in over 70 percent of virtually all major types of cancer.
DNA-based markers have advantages because of the inherent stability of DNA as compared with RNA and some proteins. DNA methylation, although being reversible, is chemically stable and many assays are available to detect hypermethylation states of the DNA. Oppositely to usual cancer genomic analysis as loss of heterozygosity where a negative result must be recognized over a positive background, the presence of methylated DNA is positively showed against a negative background.

Methylation assays can be routinely performed on biopsy samples, frozen or paraffin-embedded tissues. If performed on DNA from exfoliative tissues found in the blood or in different bodily fluids it offers the opportunity for a virtually non-invasive analysis for screening

purposes. Sensitive analysis (i.e. methylation specific PCR), which is a main requirement together with specificity to test peripheral bodily fluids, allows the detection of hypermethylation in serum, sputum, bronchial lavage fluid, urine, ductal fluids and lymph nodes from patients affected (potentially) by different types of cancer[63,64,65,66,67] and also allows to early detect (up to three years before the clinical appearance of the tumor) pre-malignancies due to the early role of methylation in tumor stages[68].

The ideal approach for this kind of analysis, which is useful either in diagnosis and prognosis, is the definition of a validated, sensitive and specific panel of markers (gene promoters), able to detect individual cancer types. Furthermore these approaches could also allow the discrimination between different forms of the same tumor. In lung tumor a five genes signature allowed to distinguish differently aggressive cancers (mesothelioma and adenocarcinoma) and healthy tissue[69].

Some criticalities of this approach need to be solved before the full potential of DNA methylation detection as cancer marker can be exploited: first the level of DNA methylation in healthy tissues must be addressed, if only related to aging or to the actual onset of a cancerous phenomenon. Further a deep study of the position of the methylation of each considered promoter in the progression of each different tumor should be performed in order to be able to well distinguish between a potential risk of cancer (pre-malignant lesions) and the actual possible presence of a cancer at early phase.

Another possible application of detection of CpG islands hypermethylation could be to gauge the prognosis of the cancer and the susceptibility to drugs. This could complement the classical evaluation of the prognosis based on the assessment of the size of the tumor, the involvement of lymph nodes and the presence of distant metastasis. For example, among the hypermethylated and silenced genes are hMLH1 and O6-MGMT, which encode DNA-repair proteins. When the function of these genes is lost, the cells have a diminished capacity to repair alkylation damage to the base guanine and they become susceptible to guanine-to-adenine mutations. This sensitizes cells to the effects of chemotherapy that depend on an alkylating mechanism. Some studies indicate that brain tumors that carry hypermethylated O6-MGMT could respond better to alkylation therapy than those that do not[70], and this could be also verified for lymphomas[71]. Hypermethylation of MGMT gene is a good predictor of response to temozolomide in glyomas[72]. Another example with prognostic importance is the gene for Death-Associated Protein Kinase (DAPK), an antiapoptotic factor[73]. Patients

with lung tumors containing a hypermethylated death-associated protein kinase gene have a shorter survival after diagnosis than those with tumors that do not[74].

Gene hypermethylation and lung cancer

Lung cancer is the leading cause of the cancer-related deaths in the world[75]. A major factor for the high mortality of lung cancer patients is the presence of metastases in approximately two-thirds of patients at the time of diagnosis[76]. It has been estimated that detection of lung cancer at earlier stages could potentially increase survival rates by 10-50 fold[77]. Lung cancer screening by chest X-ray and sputum cytology have proven ineffective in increasing patients' survival[78], leading to the search for more sensitive and specific tests that provide non-invasive methods for the detection of lung cancer-specific biomarkers at an early stage.
Methylation status may be one of these markers, since many gene promoters are found to be highly methylated in lung cancer cell. Among them are *p16*, *DAPK1*, and *GATA5* promoter.

The *CDKN2A* gene is an important tumor-suppressor gene which encodes INK4a (also known as p16) and ARF (also known as p14), and is silenced in many cancers through aberrant promoter hypermethylation. *CDKN2A* gene is inactivated at prevalence of up to 67% in adenocarcinoma and 70% in squamous-cell carcinoma of the lung, respectively[79,80,81]. p16 is an inhibitor of cyclin-dependent kinase 4 (CDK4) and CDK6, which bind cyclin D1 and phosphorylate the retinoblastoma (Rb) tumour-suppressor gene[82,83]. Therefore, INK4A contributes to the maintenance of Rb in the unphosphorylated state, which inhibits cell-cycle progression. Interestingly, CDKN2A is almost never altered in small-cell lung cancer.

Death-associated protein kinase (DAPK1) is a serine/threonine, microfilament-bound kinase involved in γ-interferon-, TNF-α- or FAS-induced apoptosis[84,85]. Loss of function of this kinase could significantly affect cellular apoptosis. Methylation of the *DAPK1* promoter is observed in both adenocarcinoma and squamous-cell carcinoma at prevalences ranging from 30–48%[86,87]. DAPK1 can then suppress oncogenic transformation of primary embryonic fibroblasts by activating p53 in an ARF-dependent manner, indicating an important role for this gene in eliminating premalignant cells during cancer development.

GATA binding proteins are a family of transcription regulatory proteins containing two conserved zinc finger DNA-binding domains recognizing

the sequence WGATAR[88,89]. GATA-1, -2, and -3 are important in the development and differentiation of the hematopoietic cell lineage[90]. GATA-4, -5, and -6 guide development and differentiation in endoderm-derived organs, including heart, lung and gastrointestinal tract[91,92]. *GATA* genes have been implicated in cancer development and lung cancer development too[93]. GATA proteins bind the promoters of a number of proposed antitumor genes, and have been suggested as transcriptional activators for these genes.

Detection of DNA methylation

A wide variety of methods have been developed to assess the methylation status of specific genes in a broad range of applications. Several of these methods were originally developed for detection of single-nucleotide polymorphisms (SNPs) and disease causing mutations in genomic DNA, but have been later adapted for detection of sequence differences between methylated and unmethylated alleles.

Methods for DNA methylation analysis can be divided into two main groups: genome-wide and gene-specific methylation analysis.

Analysis of genome-wide methylation content

Several assays are currently available for the investigation of the global levels of genome-wide methylation in DNA. If the desired measurement is only the overall content of 5meC in a genome, i.e., the ratio between total cytosine and total 5meC in a given sample, a chromatography-based method, such as reversed-phase high-performance liquid chromatography (HPLC) can be utilized.
Otherwise, if DNA methylation measurement in discrete compartments of the genome, such as CpG islands or repetitive sequences is desired, methods such as restriction landmark genomic scanning (RLGS)[94,95], differential methylation hybridization (DMH)[96,97], and bacterial artificial chromosome (BAC) arrays[98], among others, could be employed.

Techniques for gene-specific methylation analysis

While analysis of global DNA methylation content can be determined directly on crude DNA preparations, procedures for analysis of gene-specific DNA methylation patterns require initial amplification of the target sequence by polymerase chain reaction (PCR). Since DNA polymerases do not discriminate between cytosine and 5-methylcytosine,

epigenetic marks present in the genomic DNA template are not retained in the resulting PCR products but they are lost during the amplification. To solve this problem two main tools have been employed: Methylation-Sensitive Restriction Enzymes (MSREs) and sodium bisulfite treatment[99]. Both allow discrimination between cytosine and 5-methylcytosine.

MSREs

In methods that use the methylation-sensitive restriction enzymes, the same enzymes with different sensitivity to methyl group (isoschizomers) are used to discriminate between methylated and unmethylated sequences at specific sites. Methylation sensitive enzyme cuts the DNA sample only if it is methylation-free, while leaves it undigested if it presents methylated cytosine(s) into the restriction site (**Figure 9**). The big limitations of this technique are that it provides information only about CpGs within the cleavage sites of the specific enzymes, the risk of false positive due to the incomplete digestions and the requirement of large amount of DNA samples.

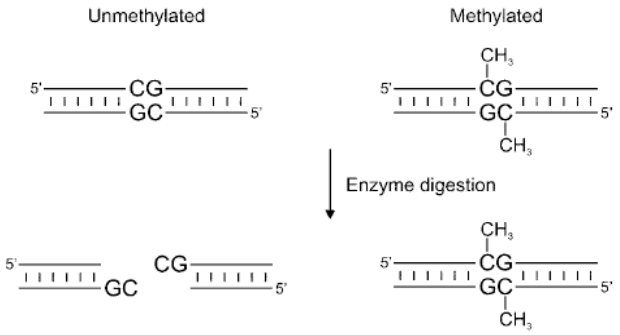


Fig. 9 Principle of methylation analysis using MSREs. Genomic DNA is digested with a restriction endonuclease that will not cut if the cytosines within the cleavage site are methylated.

The methylation sensitive digestions are usually followed by Southern blot analysis or PCR for the detection of the information of the methylation status. In the first case, digestion products are fractionated by agarose-gel electrophoresis, blotted onto a nylon membrane and then

hybridized with a radio-labeled probe specific for the gene of interest. If the investigated site is methylated, the bands generated with the methylation-sensitive isoschizomer will differ in size from those generated with the non-sensitive isoschizomer. The fraction of digestion-resistant DNA may be quantified by image analysis and is proportional to the degree of methylation present at that site (methylation level). Otherwise, if the digestion product is analyzed by PCR, primers that flank the restriction site are used, then a product will only be obtained if the site is not cleaved by the endonuclease.

Bisulfite-based methods

The analysis of DNA methylation at the level of the individual nucleotide was revolutionized in 1992 by the introduction of sodium bisulfite conversion of genomic DNA (**Figure 10**) by Frommer et colleagues[99].

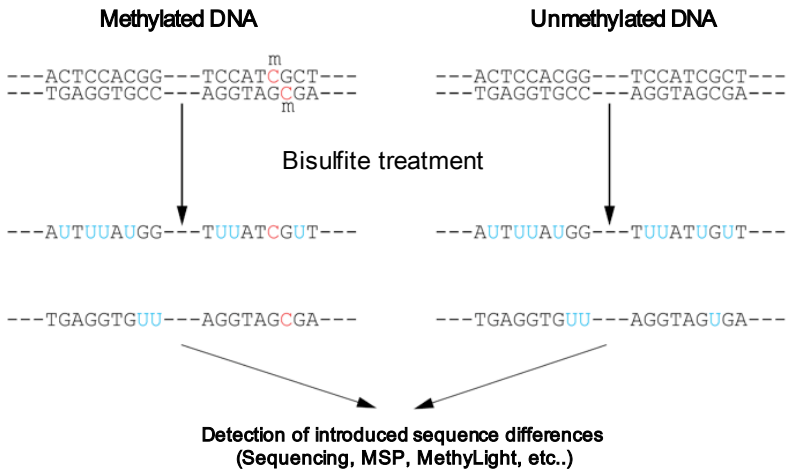


Fig. 10 Treatment of genomic DNA with sodium bisulfite deaminates unmethylated cytosine residues to uracil while 5-methylcytosine (in red) remains unaffected. After bisulfite conversion the newly inserted sequence differences can be analyzed by several different methods.

Sodium bisulfite converts unmethylated cytosines into uracils while leaving methylated cytosines intact, thus creating sequence-differences

between genomes originally differing only in their CpG methylation pattern. The nucleotide differences are then detected by sequencing[100], restriction enzyme analysis, PCR[101], and other methods.

According to the modification protocol originally developed by Frommer et al., genomic DNA is fragmented by digestion with appropriate restriction enzymes, denatured with sodium hydroxide, treated with bisulfite at pH 5, desalted and desulfonated with sodium hydroxide. Finally, the DNA is neutralized, desalted and resolved in water and results ready to further analysis techniques. Since the time Frommer et colleagues introduced the use of this chemical treatment in the laboratory practice of epigenetic studies, bisulfite-treated DNA is the starting material for most DNA methylation detection techniques.

Although sodium bisulfite conversion represents a powerful method for identification of DNA methylation, it is also a tremendously harsh treatment that results in significant levels of DNA degradation (approaching 85 to 95% after 4 hours)[102], thus introducing the problem of reproducibility and sample preservation.

Bisulfite genomic sequencing

At present, sodium bisulfite conversion of DNA followed by PCR and DNA sequencing remains one of the gold standards for methylation analysis. Although this approach provides a method for detailed analysis of DNA methylation within a given gene sequence, it is labor intensive, time consuming, relatively expensive, and not readily adaptable to high-throughput analysis of DNA samples.

Combined bisulfite restriction analysis (COBRA)

COBRA[103] exploits differences in restriction sites between methylated and unmethylated DNA samples after treatment with bisulfite (**Figure 11**). If the investigated cytosines are methylated, during the chemical conversion the CpG dinucleotides inside the restriction site remain CG, while become UG (TC during the PCR) if they are unmethylated. The sequence of interest is amplified by PCR using primers that do not discriminate between methylated and unmethylated alleles. The PCR products are then digested with an appropriate restriction enzyme that contains CG or TG in its restriction site. According to the selected enzyme, a positive result (cleavage) can indicate either non-methylation or methylation status of the site.

COBRA analysis allows the determination of DNA methylation levels at specific loci in small amounts of genomic DNA but as the other restriction enzymes-based methods, COBRA is limited to restriction sites and thus cannot be used to analyze the methylation status of all possible CpG sites.

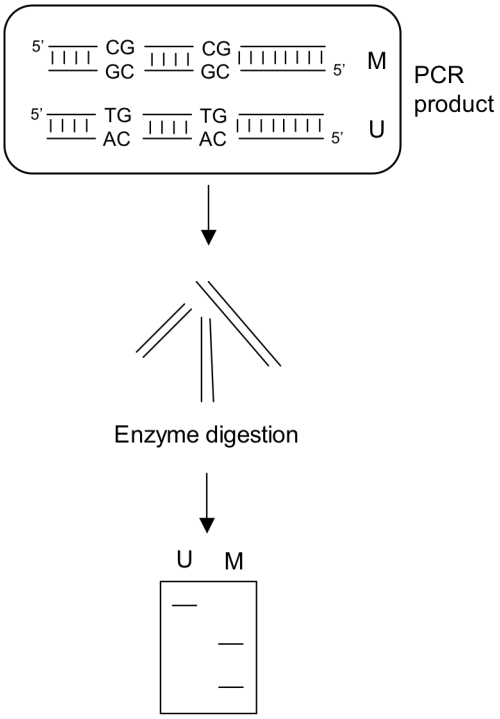


Fig. 11 In combined bisulfite restriction analysis (COBRA) PCR products are generated from bisulfite-treated DNA using primers that do not discriminate between methylated (M) and unmethylated (U) templates. Then they are digested by a specific restriction enzyme that contains CG or TG in its restriction site and the digestion products are visualized by agarose gel electrophoresis.

Methylation Specific PCR

Methylation-specific PCR is one of the most widely used technique to investigate the methylation status of specific CpG sites in CpG islands[101]. The basic principle of this method (**Figure 12**) is PCR-based discrimination between methylated and unmethylated DNA in bisulfite-converted DNA, taking advantage of the bisulfite-induced sequence

differences. Two sets of primers can be used in two separate reactions to specifically amplify methylated and unmethylated molecules, respectively, and the PCR products are then analyzed by ethidium bromide-stained agarose gel electrophoresis.

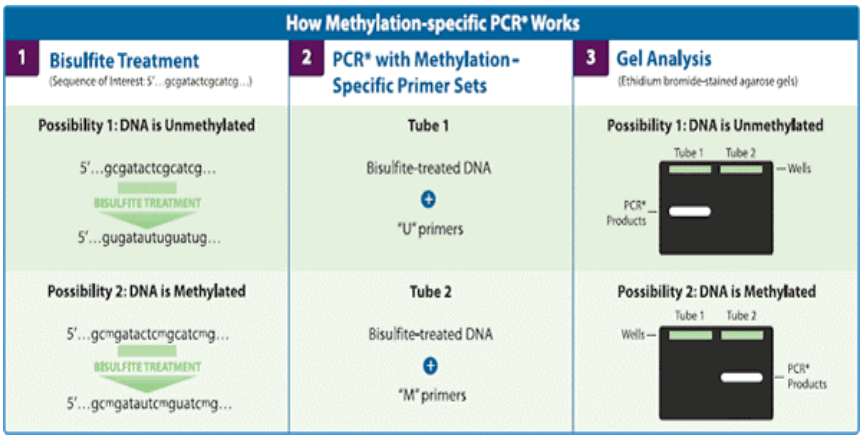


Fig.12 Schematic outline of methylation-specific PCR. Genomic DNA is treated with bisulfite (**1**) and used as template in a two PCR reaction, each containing primers designed to amplify methylated (M) or unmethylated (U) sequences (**2**). PCR products are then analyzed by ethidium bromide-stained agarose gel electrophoresis (**3**).

Methylation-specific PCR requires only small quantities of DNA as starting template. Furthermore, methylated molecules can be detected down to a level of 0.1% in the total population. Methylation-specific PCR is, therefore, best suited as a rapid and cost-effective method to initially screen samples for methylation of specific genes.

MethyLight

MethyLight[104] relies on fluorescence-based real-time PCR. After bisulfite conversion, the DNA is amplified in a taqman-based assay reaction containing three oligonucleotides, a dual-labeled probe and two primers, all of which are specific for the target (**Figure 13**). Target

sequence discrimination can be achieved at three levels: through the design of methylation-specific primers, which may or may not overlap with CpG dinucleotides; through the design of the fluorescent probe, which could overlap one or various CpG sites; or both. The probe has a 5' fluorescent reporter dye and a 3' quencher dye. When the probe is intact, that is, when there is no target amplification, the quencher dye absorbs the fluorescence of the reporter dye due to their proximity. In presence of the specific target, during the amplification process, the probe anneals to the template and is cleaved by the 5'-3' exonuclease activity of Taq DNA polymerase. This cleavage will release the reporter from the quencher, resulting in an increase in fluorescence signal. The fluorescence is measured at every cycle and it is proportional to the amount of PCR product generated. The methylation level of the starting template can be derived from the cycle number at which the fluorescent signal crosses the detection threshold in the exponential phase of the PCR reaction.

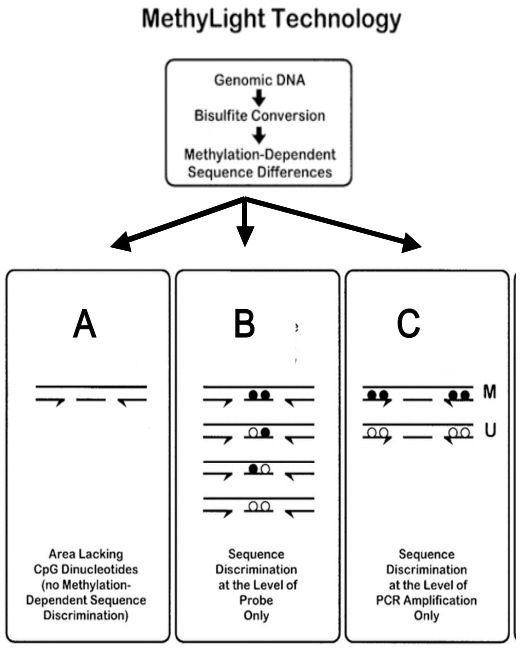


Fig. 13 Schematic of the theoretical basis of MethyLight technology. Genomic DNA is first chemically modified by sodium bisulfite. This generates methylation-dependent sequence differences at CpG dinucleotides by converting unmethylated cytosine residues (white circles) to uracil, while methylated cytosine residues (black circles) are retained as cytosine. Fluorescent-based PCR is then performed with primers that either overlap CpG methylation sites or that do not overlap any CpG dinucleotides. Sequence discrimination can occur either at the level of the PCR amplification process (**B**) or at the level of probe hybridization process (**A**), or both (**C**).

The MethyLight technique has several important advantages. Because of its reliance on PCR amplification, it is a highly sensitive assay, capable of detecting methylated alleles in the presence of a10,000-fold excess of unmethylated alleles and it is suitable for the analysis of samples where the available DNA amount may be small or not of the highest quality. Second, the risk of PCR contamination is significantly reduced because no manual transfer of PCR products is required.

MS-SnuPE

Single-nucleotide primer extension is a well-established method that has been successfully used for the detection of gene mutations. MS-SNuPE (**Figure 14**) relies on single nucleotide primer extension to assess DNA methylation at a specific cytosine. An initial round of PCR is carried out using bisulfite DNA-specific primers, followed by a second PCR step that uses a primer that terminates precisely 5′ of the nucleotide whose methylation status is unknown. At this step, radio-labeled dCTP and dTTP are added in two separate reactions. The radio-labeled products are then run on a 15% polyacrylamide gel and visualized via exposure to an autoradiographic film. The intensity of the observed bands can be then quantified to determine the proportion of C/T at the site of interest.

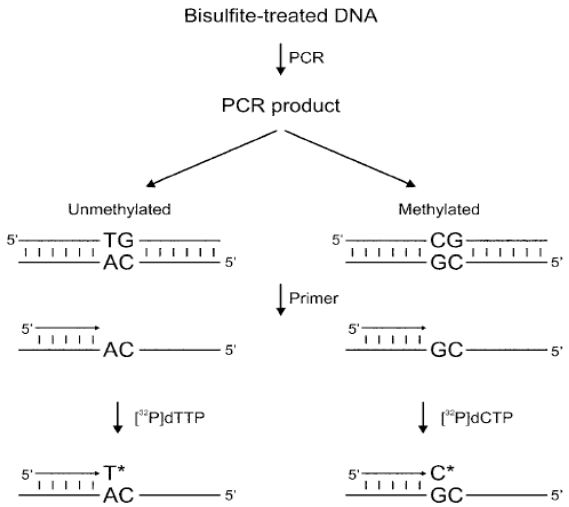


Fig. 14 Schematic outline of Ms-SnuPE. Genomic DNA is treated with bisulfite followed by PCR amplification using primers that do not discriminate between methylated and unmethylated alleles. The PCR product is then incubated with a primer that anneals to sequences upstream and terminates immediately 5' to the site being monitored. After primer annealing, a single-nucleotide extension reaction is performed in the presence of *Taq* polymerase and [α-32P]dTTP or [α-32P]dCTP. The radiolabeled reaction products are typically electrophoresed in polyacrylamide gels and quantified by phosphorimage analysis.

Pyrosequencing

Pyrosequencing is a quantitative bisulfite sequence-by-synthesis approach that is based on the luminometric detection of pyrophosphate release following nucleotide incorporation[105,106]. Pyrophosphate converts to ATP, which provides the energy to luciferase to oxidize luciferin and generate light. Nucleotides are added sequentially to enable base calling. If the added base is not the proper Watson–Crick partner no luminescence is detected and the base is removed by washing.

The major advantage of the pyrosequencing method compared to PCR-based amplification methods is that its output consists of nucleotide sequences rather than data from fluorescence or PCR band in agarose gel. However, its major drawback is that it requires quite expensive dedicated equipment.

Microarray

Traditional PCR-based techniques for detection of DNA methylation are best suited for the analysis of single or a small number of genes. In recent years, however, research studies have focused on the concomitant investigation of DNA methylation in a relatively large number of genes[107]. Oligonucleotide-based microarrays for DNA methylation analysis uses bisulfite-treated DNA as template for non-discriminatory PCR amplification, followed by hybridization of the PCR product to glass slides carrying oligonucleotides that discriminate between methylated and unmethylated cytosines at specific CpG positions[108] (**Figure 15**).
To allow detection of hybridization signals by fluorescence analysis, the amplification primers carry a fluorescent label. One of the great potentials of the use of microarray for the detection of DNA methylation is that multiple genes can be analyzed on the same array. For example, Adorjan et al. (2002) assessed the methylation status of 232 individual CpG sites representing 56 different genes as a means to distinguish different solid tumors. Oligonucleotide arrays have been successfully used to characterize the methylation profile of non-Hodgkin's lymphomas and breast tumors[109,110].

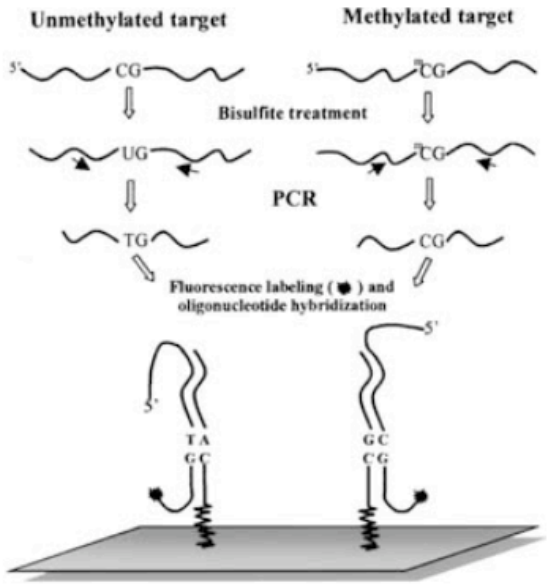


Fig. 15 Schematic outline for analysis of DNA methylation based on oligonucleotide microarray. Genomic DNA is bisulfite treated an amplified by PCR for a specific CpG island region of interest. The amplified product is labeled with Cy5 fluorescence dye and hybridized to oligonucleotide probes attached to a glass surface. At left an oligonucletide probe is designed to form a perfect match with a target DNA containing the unmethylated allele. At right a probe is designed to form a perfect match with the methylated DNA target

Quantitative DNA hypermethylation detection

A number of quantitative MSP techniques have been developed in recent years to evaluate relative amounts of DNA promoter hypermethylation in a given sample.

Quantitative information in real-time methylation assays are usually reached by parallel amplification of an internal reference gene for the input DNA, usually the beta-actin gene (*ACTB*), the *GAPDH* or the myogenic differentiation antigen 1 (*MYOD1*), that allows relative quantification of the methylation sites.
Gene reference primers are designed for a stretch of the reference gene completely devoid of CpG dinucleotides in order to obtain a methylation-independent amplification for the internal reference standard whereas the amplification of the tested gene is proportional to the degree

of cytosine methylation. Serial dilution of Sss1 treatment (which converts all cytosines to their methylated forms) of human genomic DNA is used to create a standard curve. The fluorescence emission intensities (threshold cycles or Ct values) for the biomarker gene and reference are calculated using the intercept and the slope of the standard graph. The methylation ratio is defined as the ratio of the fluorescence emission intensity values for the PCR products of the biomarker gene to those of PCR products of reference gene multiplied by 100.

$$\% \, M = \frac{\text{Ct biomarker}}{\text{Ct reference gene}} \times 100$$

The ratio is a measure for the relative level of methylation in an individual sample.

Relative quantitation of gene methylation is also achieved by parallel amplification of the methylated promoters and their unmethylated counterpart sequence, using primers that carry T (or A in the opposite strand) instead of C (or G, in the opposite strand), in this case the M level is expressed as:

$$\% \, M = \frac{\text{cps M}}{\text{cps (M+U)}} \times 100$$

The numbers of copies of methylated and unmethylated DNA are extrapolated from the respective standard curves.

Real-time approaches such as MethyLight can use two reaction wells per sample to characterize methylation of a single gene. The methylation reaction is carried out in one well, while a reference gene, such as actin, is amplified in a second well. Similarly, the gel-based MSP methods of Herman et al. and the real-time QM-MSP method of Fackler et al.[111] use one well for primers specific to the unmethylated U gene fraction and a second well for primers specific to the methylated M gene fraction.

Loop-Mediated Isothermal Amplification

In the last years many different DNA amplification techniques have been developed, showing performance comparable to PCR but peculiar characteristics which make them potentially advantageous for molecular diagnostics applications. One of these techniques is the Loop-Mediated Isothermal Amplification (LAMP)[112].
LAMP is an isothermal DNA amplification method which relies on the use of a thermostable DNA polymerase with strand displacement activity (*Bst* polymerase from *Bacillus stearothermophilus*) and on 6 primers designed in a peculiar way, in order to anneal to 8 distinct areas of a chosen region of a target DNA (**Figure 16**).

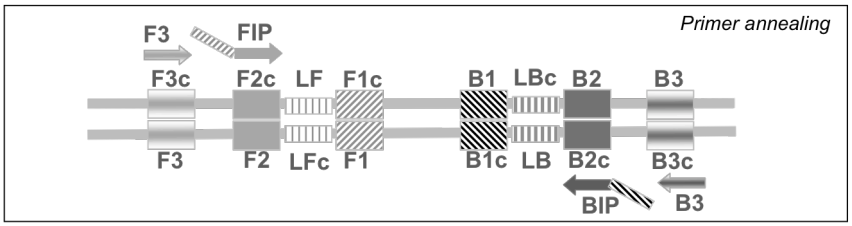


Fig. 16 Outline of LAMP primers placement. FIP and BIP anneal respectively on F2c and B2c regions; their tails anneal on the F1 and B1 regions sited on the extension products of FIP and BIP on their target. These DNA strands are displaced by F3 and B3 extension on the target, leading to the following step. LF and LB regions are indicated as the target regions for Loop Primers annealing.

Through a specific dynamic of annealing and displacement a "dumb-bell" starting structure is obtained from FIP, BIP, F3 and B3 primers. This DNA structure contains a double stem-loop which is the starting point for the whole LAMP amplification process as it promotes an amplification from its self-annealed 3' terminus and from a newly annealed internal primer (FIP or BIP). Moreover it contains single-stranded regions targeted by Loop Primers (**Figure 17**).

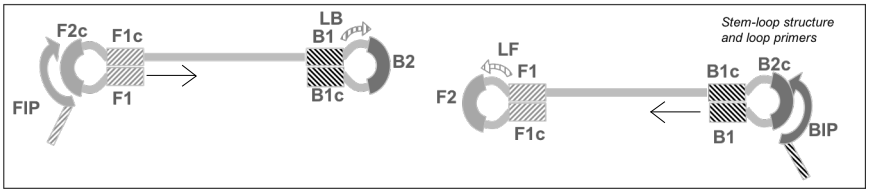


Fig. 17 Stem loop structures. "Dumbbell" structures are generated by FIP, BIP, F3 and B3 activity and they promote the following exponential-like amplification. LF and LB primers anneal on a single stranded region and co-operate to the amplification of DNA.

LF and LB primers are defined as loop primers and act as reaction boosters annealing and extending on single strand regions on the loop regions of the dumb-bell structure. Their presence increases the rate of DNA production and the sensitivity of the method and decrease the reaction time.

The main reaction products are double strand DNA molecules of variable length, composed by inverted repeats concatamers of the initial module identifiable on the target in a region comprised within F2 and B2 (**Figure 18**).

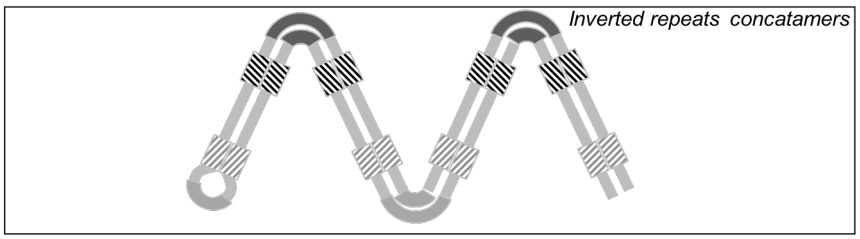


Fig. 18 Reaction products. Inverted repeats concatamers are the main amplification products of a LAMP reaction.

By these peculiar amplification dynamics and exploting the continuous displacement by Bst polymerase of the already produced DNA strands with no need for denaturation steps, it is possible to amplify the initial DNA amount up to 10^{10} times in less than 60 minutes and with very high specificity, due to the many independent target-recognition events.

Reaction results are detectable either in a fluorescent and a turbidimetric way. Many fluorescence approaches are employable, like intercalating dyes or molecular beacons and quenching probes which allow multiplex assays when different fluorophores (specific for distinct targets) are used at the same time.

Turbidimetry detection is made possible by the high amplification efficiency of LAMP reaction[113,114]. This detection method consists in the measurement of turbidity of the reaction mix, due to the precipitation of magnesium pyrophosphate. This insoluble salt is formed by the interaction between Mg^{2+} present in solution and the inorganic pyrophosphate produced by the incorporation of dNTPs in the DNA growing strands. Thus, magnesium pyrophosphate presence is the indication of the occurrence of the amplification reaction and its amount is proportional to the amount of amplified DNA and it is measurable as a trasmittance signal either at end-point for a qualitative analysis and in real-time, also allowing quantitative applications.
Turbidimetry is the ideal detection method for LAMP technology, at least for fast clinical applications and point-of-care approaches since it does not require expensive fluorescent reagents and complex detectors.

One more possible, very easy, detection approach can rely on fluorescent dye calcein and again it exploits the high reaction efficiency of LAMP: this chemical compound, that can be included in the reaction is complexed with manganese ion and in this state its fluorescence is quenched. When the high amount of inorganic pyrophosphate is produced by the reaction, it complexes the Mn^{2+} ion instead of Mg^{2+} and calcein emits. Emission of calcein can be very easily detected by irradiating the tubes with a standard transilluminator or even by naked eye (**Figure 19**).

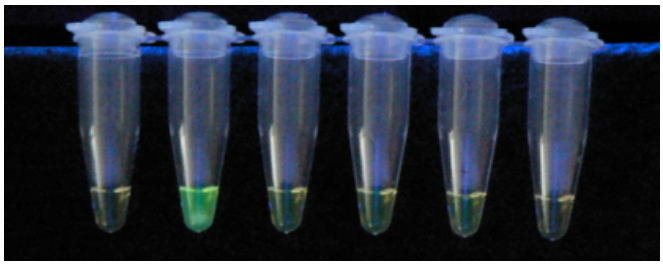


Fig 19 LAMP reaction revealed by calcein. Calcein included in the reaction mix allows an easy detection by a common UV-transilluminator. Its fluorescence emission is promoted by PPi produced during the amplification.

Thus LAMP constitutes an attractive alternative to PCR for sequence detection, with a sensitivity and quantitative performance comparable to PCR, with extraordinary specificity, allowing different detection methods, simplex and multiplex reactions applications in a fast, easy and potentially cheap molecular diagnostics platform. Moreover a RT (reverse transcription) LAMP was developed for the detection and amplification of RNA[115,116], thus moreover increasing the fields of applications for this technique.

LAMP has been explored so far in hundreds of papers for the detection of the DNA of a great amount of different organisms, such as bacteria[117,118,119], viruses[120,121,122], fungi[123,124], and parasites[125,126,127], but it seems also suitable for the analysis of human DNA, i.e. for the detection of pathological determinants as SNPs and deletions. Encouraged by the evidence that the LAMP technology was used successfully in these applications requiring a high specificity, we decided to challenge the potential of the technology for the detection of DNA hypermethylation.

AIM OF THE THESIS

There is today strong evidence that epigenetic changes are as important as genetic modifications in the tumorigenesis processes and seem to be involved in the earliest steps of cancer progression. Detection of aberrant status of methylation of key gene promoters, both in the location of the tumor and in different bodily fluids, could help in the early diagnosis of these serious pathologies. Moreover, prognosis information and therapeutic indications could be obtained by monitoring the methylation status of an accurately chosen pattern of gene promoters. A sensitive and specific technique is necessary for this kind of analysis.

Many methods have been developed so far, but some of them need labor intensive procedures, provide a partial information or require expensive apparatus and reagents.

The aim of this work is to develop a novel method for the detection of DNA methylation, based on a combination of bisulfite treatment and Loop-mediated Isothermal Amplification. This is a new method for DNA amplification, already explored as a promising platform for many molecular diagnostics applications, characterized by high specificity, short reaction duration, performance comparable to PCR methods (in terms of sensitivity and quantitative capability) and ease and flexibility in reaction chemistry and detection systems. Three MS-LAMP assays were designed to detect methylation status of three gene promoters related to onset of lung tumor and performance of the assays were tested on different types of controls and clinical samples. A multiplex approach was also explored, for a fast screening of a methylation genes pattern.

MATERIALS AND METHODS

Target DNA

Plasmids

For a preliminary test of the reliability and the performance of LAMP assay without the variability brought by bisulfite treatment, we performed specificity, sensitivity and selectivity experiments onto plasmids incorporating the sequence of DAPK promoter either in its methylated or unmethylated state after a bisulfite treatment. P16 and GATA5 methylated and unmethylated plasmids were also employed as controls for multiplex experiments. Plasmids were synthetically generated by Geneart AG. Unmethylated and methylated sequences virtually treated by bisulfite are obtained substituting respectively all the cytosines or all the cytosines except those included into CpG dinucleotides with tymines. A synthetic double strand DNA sequence is automatically synthesized by the supplier company and it is cloned in a plasmid which is then amplified by *E. coli* transformation. Finally the plasmid is extracted and sent to the customer with a very high degree of purity. Sequences of the "unmethylated" and "methylated" plasmids were as follows

pDAPK U

```
TGGAGTGTGAGGAGGATAGTTGGATTGAGTTAATGTTGGGGATTTTGTT
TTTTTTGTGGAGGGGATTTGGTAATTTGTAGTGGTAGGGTTTGGGGTTG
GTGTTTGGGAGGGATTTGTGTTTTTTATTTATTTTTTAGTTGTGTTTTT
GTTGTTGTTTTGGTTAGTTTTTGGTGTTGGTGTTTATGGTTGGTTTTTG
ATAGTGTTTTGGAGGG
```

pDAPK M

```
CGGAGTGTGAGGAGGATAGTCGGATCGAGTTAACGTCGGGGATTTTGTT
TTTTTCGCGGAGGGGATTCGGTAATTCGTAGCGGTAGGGTTTGGGGTCG
GCGTTTGGGAGGGATTTGCGTTTTTTATTTATTTTTTAGTTGTGTTTTC
GTCGTCGTTTCGGTTAGTTTTCGGCGTTGGCGTTTATGGTCGGTTTTCG
ATAGCGTTTCGGAGGG
```

pp16 U

ATTAGAGGGTGGGGTGGATTGTGTGTGTTTGGTGGTTGTGGAGAGGGGG
AGAGTAGGTAGTGGGTGGTGGGGAGTAGTATGGAGTTGGTGGTGGGGAG
TAGTATGGAGTTTTTGGTTGATTGGTTGGTTATGGTTGTGGTTTGGGGT
TGGGTAGAGGAGGTGTGGGTGTTGTTGGAGGTGGGGGTGTTGTTTAATG
TATTGAATAGTTATGGTTGGAGGTTGATTTAGG

pp16 M

ATTAGAGGGTGGGGCGGATCGCGTGCGTTCGGCGGTTGCGGAGAGGGGG
AGAGTAGGTAGCGGGCGGCGGGGAGTAGTATGGAGTCGGCGGCGGGGAG
TAGTATGGAGTTTTCGGTTGATTGGTTGGTTACGGTCGCGGTTCGGGGT
CGGGTAGAGGAGGTGCGGGCGTTGTTGGAGGCGGGGGCGTTGTTTAACG
TATCGAATAGTTACGGTCGGAGGTCGATTTAGG

pGATA U

TTTTTTTTTTTGTGTTTGTTTTTTTTTTTTTGTAGGGTTAGTGTTGGGG
TTTTGGTTGTAGAGTTATGTGATTTTGGTAGGTTTTGTTTGTGGGGTTT
GGTGATAAGGATGTATGATATGGGGTGGTTAGTGTGGAGTTTGGATTAG
TGTAGTTAGATGGGTGGGTTGAGGGTTTTGTGGGTTTTGGGGAGTTGGG
TGTAGAGATTTTTTTAAGTTTGTGTGAGTTTTGAGTTTTTTG

pGATA M

TTTTTTTTTTCGCGTTCGTTTTTTTTTTTTCGTAGGGTTAGCGTTGGGG
TTTCGGTCGTAGAGTTACGTGATTTTGGTAGGTTTTGTTCGCGGGGTTT
GGCGATAAGGACGTACGATACGGGGCGGTTAGCGCGGAGTTCGGATTAG
TGTAGTTAGACGGGCGGGTCGAGGGTTTCGTGGGTTTCGGGGAGTCGGG
CGTAGAGATTTTTTTAAGTTTGTGCGAGTTTCGAGTTTTTTG

Genomic DNA

The fully methylated and unmethylated human genomic DNA used as positive and negative control in our experiments were CpGenome Universal Methylated and Unmethylated DNA (Millipore). Methylation status of these genomic DNA controls are guaranteed by the supplier: unmethylated DNA usually comes from a primary human fetal cell line and is assayed for methylation status of many promoters of interest from the company itself . Fully methylated DNA is obtained by the enzymatic treatment of unmethylated DNA by Sss1 methylase which links a methyl-group to every cytosine included in a CpG dinucleotide.

Clinical samples

Lung adenocarcinoma tumor samples were obtained by Massachusetts General Hospital Tumor Bank, Boston, after having obtained internal review board approval. DNA was extracted from the samples using the Dneasy Tissue Kit (Qiagen).

Bisulfite treatment

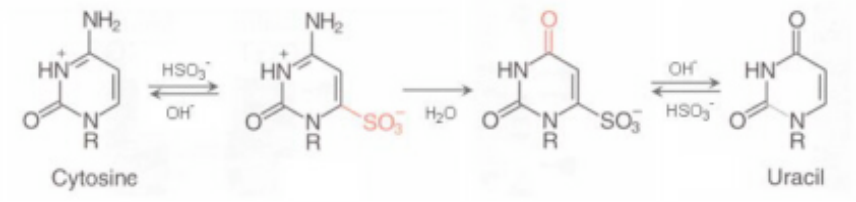


Fig 20 Steps for bisulfite modification of DNA. When DNA is treated with sodium bisulfite unmethylated cytosines are first sulfonated, then deaminated and in the end desulfonated, to obtain uracils that, in a DNA amplification reaction, behave like thymidines.

Conversion of all the unmethylated cytosines to uracils (working as thymines in amplification reactions) was obtained treating 500 ng of genomic DNA controls and of lung adenocarcinoma samples DNA with sodium bisulfite using the EZ-DNA kit (Zymo Research). This kit, based on purification columns, allows the treatment either starting from tissues or from purified DNA, as it can integrate a tissue lysis step at the beginning of the procedure and it is much more easy to use and time-saving than many other commercial kits and home-brew protocols . In our case we always started from 500 ng of purified DNA, which is the highest suggested DNA amount to obtain an optimal conversion and purification yield. DNA is incubated in a thermo-cycler for 8 minutes at 98°C and for 3.5 hours at 64°C with the "CT" conversion reagent: during this step the DNA is denatured and cytosines are converted into sulfonated uracils. The DNA is then adsorbed to a specific resin packed in a micro-column and, after a washing step with ethanol, a desulfonating agent is added directly to the column for 18 minutes in order to detach the sulfo-group to obtain regular uracils. After two washing steps final

elution of the DNA is performed in at least 10 μl of provided elution buffer or TE with a pH≥6. In our case elution was done with 20 μL of TE buffer (pH 8.0). 1 or 2 μL (approximately 25-50 ng) of DNA were used as target for hypermethylation status investigation of CDKN2A, DAPK and GATA5 promoters in our MS-LAMP or in MS-PCR.

LAMP assays

LAMP primers (F3, B3, FIP, BIP, LF, LB), specifically designed to amplify only the methylated sequence of CDKN2A (p16), DAPK and GATA5 promoters after bisulfite treatment, were designed with the support of Primer Explorer IV (Eiken, Japan).

Primers were synthesized by MWG Biotech. Their sequences are listed in Table 1

CDKN2A	F3	TGGGGCGGATCGCGTG
	B3	ACCGTAACTATTCGATACGTTA
	FIP	CCCGCCGCCGACTCCATACTACTTCGGCGGTTGCGGA
	BIP	GGAGTTTTCGGTTGATTGGTTGGTCCCCCGCCTCCAACAA
	LF	CGCTACCTACTCTCCCCCTC
	LB	GGGTCGGGTAGAGGAGGTG
GATA5	F3	TCGTAGGGTTAGCGTTGG
	B3	CTCGAAACTCGCACAAAC
	FIP	TAACCGCCCCGTATCGTACGTCGTCGTAGAGTTACGTGATTTTGG
	BIP	GAGTTCGGATTAGTGTAGTTAGACGGTCTCTACGCCCGACTCC
	LF	GCCAAACCCCGCGAACA
	LB	GGTCGAGGGTTTCGTGGGT
DAPK	F3	GGAGGATAGTCGGATCGAGTT
	B3	CGCTATCGAAAACCGACCA
	FIP	AACGCCGACCCCAAACCCTACGGATTTTGTTTTTTTCGCGGA
	BIP	GGGAGGGATTTGCGTTTTTTATTCCAACGCCGAAAACTAACC
	LF	CGCTACGAATTACCGAATCCC
	LB	AGTTGTGTTTTCGTCGTCGTT

Table 1 Nucleotide sequences of the primers used for MS-LAMP assays.

Simplex LAMP reactions were performed at 63°C (DAPK, GATA5) or 65°C (p16) for 60 minutes and monitored in a Real Time Turbidimeter LA-200 (Teramecs). The real-time turbidimeter is a relatively simple and inexpensive instrument; each of the 32 wells hosted in the instrument has

an LED as light source and a detector which measures every 6 seconds the variation in light transmittance through the test tube in which the LAMP reaction occurs. Light transmittance decrement is due to precipitation of the insoluble salt generated by the interaction between Mg^{2+} present at high concentration (8mM) in the reaction mix and the pyrophosphate which is continuously produced as a side product of the incorporation of dNTPs in the growing DNA strands. DNA amplification is graphically represented plotting arbitrary units of turbidity measured in real time against reaction time.
Reaction mixtures contained 200 nmol/L of F3 and B3 primers, 800 nmol/L of LF and LB primers, 1.6 mmol/L of FIP and BIP primers, 1.4 mmol/L of each dNTP, 0.8 mol/L betaine, 20 mmol/L Tris-HCl, 10 mmol/L KCl, 8 mmol/L $MgSO_4$, 10 mmol/L $(NH_4)_2SO_4$, 0.1% Tween 20, 0.32 U/mL Bst polymerase (New England Biolabs).

In experiments performed to assess the sensitivity and selectivity of the new method on plasmid targets a DNA denaturation preceded the LAMP reaction: this was done to better simulate bisulfite treated DNA which is partially single stranded due to loss of complementary base interaction generated by conversion of cytosines; plasmid aliquots were heated at 95°C for 10 minutes, rapidly cooled in ice for 10 minutes and finally added to reaction mix immediately before the reaction.

Multiplex MS-LAMP

Multiplex MS-LAMP – Turbidimetry

Multiplex (triplex) LAMP reactions in turbidimetry were performed at 63°C using the reagents at the same concentrations as in simplex and gathering all the 18 primers in the same reaction mix. This reaction temperature was chosen because it was the optimal one of two of the three assays while being an acceptable temperature also for the third (CDKN2A).

Multiplex MS-LAMP – Fluorescence

For the multiplex LAMP reactions in fluorescence, some of the reagents were different and some concentrations were optimized in order to allow a balanced amplification time for the three assays. LF primer of DAPK

assay was substituted with an oligonucleotide carrying the same DNA sequence but linked at its 5' with BodipyFL dye, a green emitting fluorescent dye with a maximum in emission at 511 nm. LF primer of CDKN2A assay was also substituted with an oligonucleotide carrying the same DNA sequence but marked at its 5' with TAMRA dye, a yellow emitting dye with a maximum in emission at approximately 580 nm. Finally LF primer of GATA5 assay was substituted with an oligonucleotide marked at its 5' with Bodipy 650/665, a member of the Bodipy family with an emission peak centered at 665 nm and with the following sequence, slightly shifted comparing to the original one to allow the fluorescent dye to be closer to guanosinic nucleotides: CAAACCCCGCGAACAAAA.
The described dyes were chosen to maximize the amount of quenching for each assay and to reduce to the minimum a cross-talk between the fluorescence channels which could generate false results: disposition of excitation (dotted lines) and emission (full lines) peaks of BodipyFL (green), TAMRA (blue) and Bodipy 650/665 (red) on the visible light spectrum is shown in **Figure 21**.

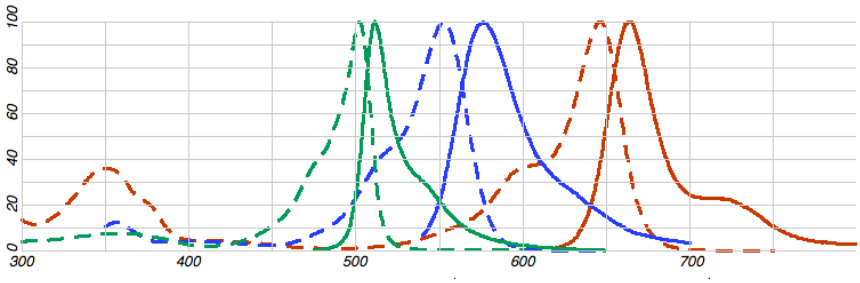


Fig 21 Excitation and emission peaks of the dyes used for fluorescence multiplex reactions. Green = BodipyFL, Blue = TAMRA, Red = Bodipy 650/665

Multiplex reactions were run in a real time thermocycler (MJ-BioRad Chromo4) under isothermal conditions and fluorescence emission was monitored in the three channels corresponding to the peaks of emission of the dyes. After the completion of the reaction the raw data were exported and elaborated with a custom Macro application for MS Excel which, starting from the raw fluorescence signal, normalizes the data and converts automatically the amount of fluorescence into percentage of

quenching, i.e. the amount of quenched fluorescence emission in each moment compared to the total quenching of the dye reached at the end of each individual reaction.

Reaction mixture contained p16 and GATA primers sets at standard concentrations and DAPK1 primer set at 0.4x concentration comparing to standard concentration (F3 and B3 at 0.08 mM, FIP and BIP at 0.64 mM and fluorescent primer and LB at 0.32 mM); 1.4 mmol/L of each dNTP, 0.8 mol/L betaine, 20 mM Tris-HCl, 10 mM KCl, 8 mM $MgSO_4$, 10 mM $(NH_4)_2SO_4$, 0.1% Tween 20, 0.64 U/ml Bst polymerase.

MS-PCR

Methylation status was also assayed by MS-PCR, using external LAMP primers (F3 and B3) as reaction primers. Reaction mixes for all three MS-PCR assays contained GoTaq polymerase (Promega) as indicated by producer, 1X provided reaction buffer, dNTPs (0.2 mmol/L each), additional $MgCl_2$ (2.5 mmol/L) and 250 nmol/L of each primer.
Protocols for PCR reactions were the following. **p16**: initial denaturation at 94°C for 2 min, 50 cycles (94°C for 30 sec, 65°C for 30 sec, 72°C for 30 sec); **DAPK**: initial denaturation at 94°C for 2 min, 45 cycles (94°C for 30 sec, 68°C for 30 sec, 72°C for 30 sec); **GATA**: initial denaturation at 94°C for 2 min, 50 cycles (94°C for 30 sec, 66°C for 30 sec, 72°C for 30 sec). Amplification products were then run in a 2% agarose gel which was then visualized in a UV-transilluminator equipped with a camera. Melting curves were generated in a real-time PCR instrument, using LC-Green (Idaho Technology) as intercalating dye, between 65 and 95°C. MS-PCR amplicon sequencing analyses were performed providing to the sequencing facility either F3 or B3 primer and preceded by a column PCR purification step (Qiagen) to ensure a better quality sequence.

RESULTS

MS-LAMP principle and primers design

MS-LAMP (Methylation Specific-Loop Mediated Isothermal Amplification) is a novel isothermal DNA amplification method for the detection of DNA methylation.

Amplification is performed using a set of primers specifically designed for methylation detection and a thermostable DNA polymerase, *Bst* polymerase, which shows a high strand displacement activity that allows a continuous amplification of DNA by the formation of characteristic loop structures; no denaturation steps are needed and reaction is carried out at isothermal conditions.
As previously mentioned (see Introduction) the primer set for a basic LAMP reaction consists of 4 primers (F3, B3, FIP, BIP) annealing to the target region in a peculiar way that allows the formation of an initial stem-loop structure and of subsequent elongated DNA strands consisting in concatamers of inverted repeats of a basic module. Loop primers (LF and LB) play an additional role by further promoting the amplification of the DNA strands and increasing the reaction efficiency.

Primers design is a key issue for the development of a LAMP assay and especially for those dedicated to methylation detection. We designed LAMP primers assisted by a software, available online at http://primerexplorer.jp/elamp4.0.0/index.html. Once the desired target sequence is uploaded and specific thermodynamic parameters are properly set, the software automatically generates a variable number of primers sets spanning the whole uploaded region. We then select few candidate sets of primers based on thermodynamic parameters and according to empiric rules suggested by the inventors of the LAMP technology.
Selected primers sets were subsequently evaluated experimentally in order to select the best performing. Primer sets devoted to detection of promoters methylation of the three genes of interest were designed to anneal to the anticipated sequence of the hypermethylated version of the promoter regions after the treatment with sodium bisulfite. Thus, the sequence of the three genes initially uploaded in the software for primers design was the one deriving from NCBI database where all the Cs were substituted by Ts except for those included in CpG dinucleotides, considered fully methylated and therefore protected from the conversion.

Among the sets proposed by the software, those chosen for experimental screening needed to meet two main requirements: first, primers were requested to anneal to target regions including the maximum possible number of CpGs, to allow the best discrimination between methylated and unmethylated promoter. In fact when the primer set reacts with an unmethylated target (where also the cytosines contained in CpG dinucleotides undergo conversion to uracils), the Tm of each primer on the aspecific target decreases depending on the number of mismatches per primer, impairing the annealing of the primer set onto the aspecific target and the amplification, thus allowing the discrimination between methylated and unmethylated DNA. The second requirement was that any of the primers of a set should not include CpGs within three bases from the extensible end of each primer, i.e. 3' of F3, B3, F2, B2, LF and LB and 5' of F1 and B1. In cancer clinical samples, promoters are frequently only partially methylated, i.e. not all the CpGs are correctly methylated. In this case, if an unmethylated dinucleotide is located in correspondence to the extensible end of a primer, the probability of a missed amplification is higher than when the same mismatch is located far from the extensible end, due to the hindrance of the mismatching bases to the polymerase activity. This could lead to false negative results which we tried to limit by excluding discrimination spots (CpGs) from the extensible ends of the primers. Even without the high contribution in terms of discrimination given by extensible ends of the primers, high specificity is conferred by the distribution of the discrimination events on 6 primers (8 regions) which need to operate on a DNA target at the same time. For each of the chosen promoters (CDKN2A, DAPK and GATA5) a certain number of primers sets were synthesized and experimentally tested for a preliminary general screening where specificity, reaction speed and reproducibility were evaluated; after this first rough evaluation, one set of primers for each assay was selected and used for all the following experiments. A summary of how each primer set maps on the promoters regions (in the methylated and bisulfite converted sequence version) is showed in **Figure 22**.

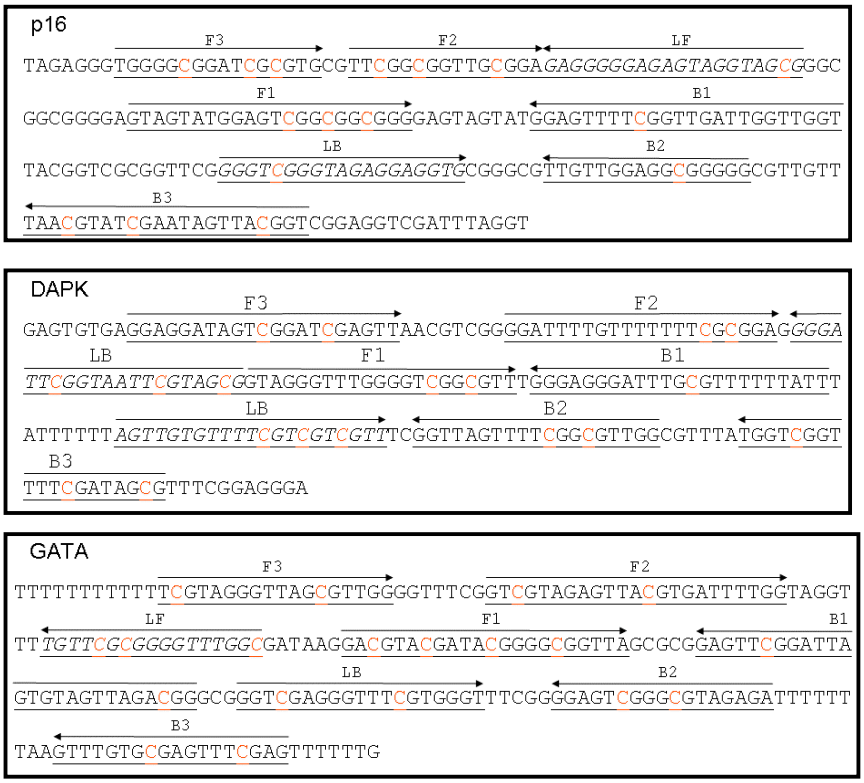


Fig 22 Outline of the position of MS-LAMP primers of each assay on the respective targets. Primer position for each assay on the respective target is showed. Target is depicted as hypermethylated and subsequently bisulfite-treated, so that all CpGs are protected from conversion. Cytosines included in the primers as discrimination spots are highlighted in red and loop primers regions are indicated in italic.

Validation of MS-LAMP principle on plasmid controls

To initially verify the base principle and to test specificity and performance of MS-LAMP for hypermethylation detection, we first synthesized two synthetic plasmids containing the anticipated sequence that the DAPK promoter region would have following complete bisulfite conversion of fully methylated (positive) or completely unmethylated (negative) sample. In the plasmid representing the fully methylated sequence all the cytosines of each CpG are kept as cytosines while the other cytosines are replaced by thymines; in the plasmid representing the unmethylated sequence after bisulfite treatment every cytosine is replaced by a thymine. Due to the variability and the low yields of the bisulfite conversion and of the subsequent purification of the DNA, these DNA controls allow a preliminary and independent evaluation of the performance of MS-LAMP without performing the chemical treatment.

Specificity

LAMP amplification curves obtained by real-time turbidimetry on 15000 copies/reaction of synthetic plasmid controls are shown in **Figure 23**. 15000 copies represent approximately the number of copies of a gene contained in 50 ng of genomic DNA per reaction.
Amplification occurred exclusively using the plasmid representing methylated DAPK promoter as reaction target while the plasmid containing the unmethylated sequence and no-target-controls (NTC, water) produced no amplification within one hour reaction.
These results indicate that MS-LAMP can discriminate sequences based on their original difference in methylation status of CpG islands.

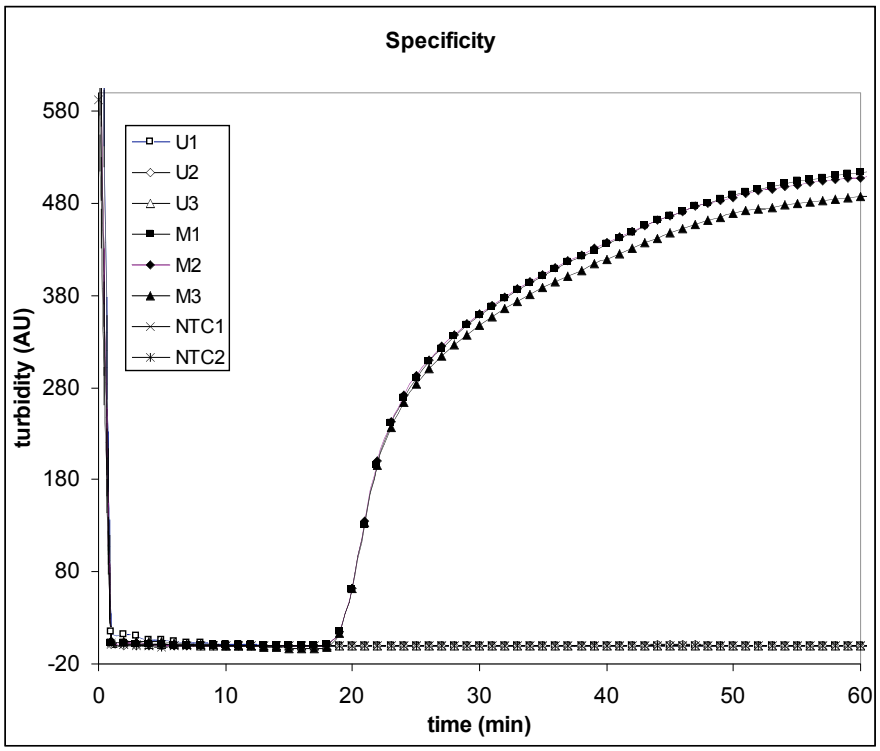


Fig. 23 Validation of MS-LAMP on synthetic plasmids: specificity. Real time amplification curves of triplicate experiments on fully methylated (U) or fully unmethylated (M) control plasmid DNA samples. Amplification is obtained only in the samples mimicking methylated DNA.

Sensitivity

In order to evaluate the sensitivity of the method, we performed MS-LAMP reaction on 10-fold serial dilutions of methylated plasmid from 100 fg/rxn (approx. $3*10^4$ cps/rxn) to 10 ag/rxn (approx. 3 cps/rxn) (**Figure 24**). Amplification was reproducibly obtained down to 100 ag/rxn (approximatively 30 copies/reaction). Semilogarithmic graph, (inset of figure 24) where the threshold time, i.e. the reaction time corresponding to the threshold value of turbidity of 100 AU, is plotted against the base-10 logarithm of the DNA amount, showed also a good linearity (R^2= 0.98).

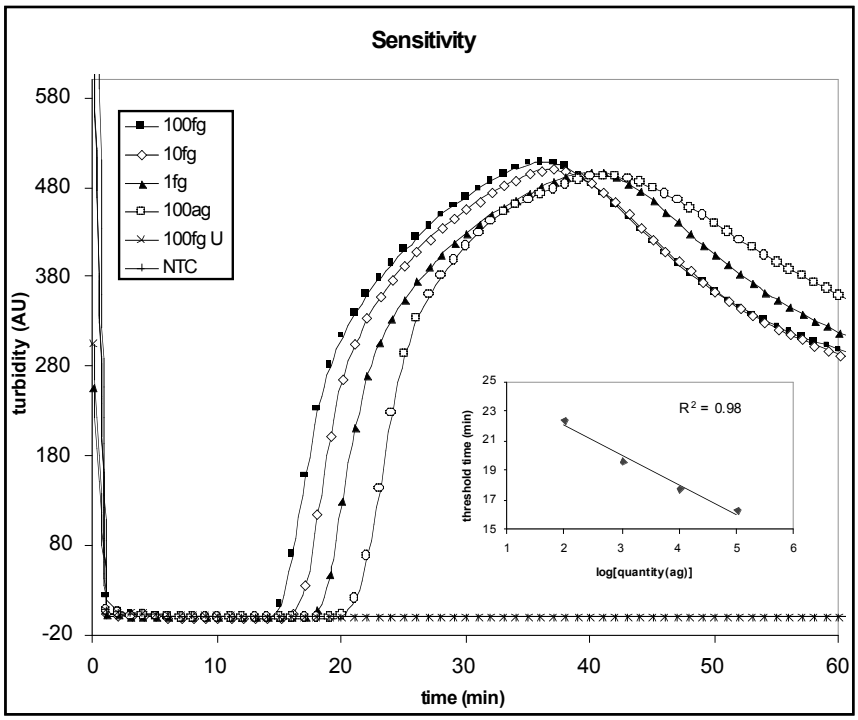


Fig. 24 Validation of MS-LAMP on synthetic plasmids: sensitivity. The assay was performed on samples containing decreasing amounts of DAPK methylated plasmid DNA. Amplification was reproducibly detected down to 100ag (approx. 30 copies) per reaction with a good linearity, R^2=0.98 (*inset*).

Selectivity

Selectivity is defined as the ability of the method to detect the specific target in presence of a high background constituted by the aspecific target. Thus we performed MS-LAMP on dilutions of methylated plasmid (M) in an unmethylated plasmid (U) background, ranging from 100%M to 0.01%M in U plasmid. Total DNA amount in each target mix was 100 fg/reaction. MS-LAMP was able to reproducibly detect down to 0.1% M plasmid diluted in U plasmid (**Figure 25**) and the detection showed a quantitative pattern (R^2=0.97) (Figure 25, inset).

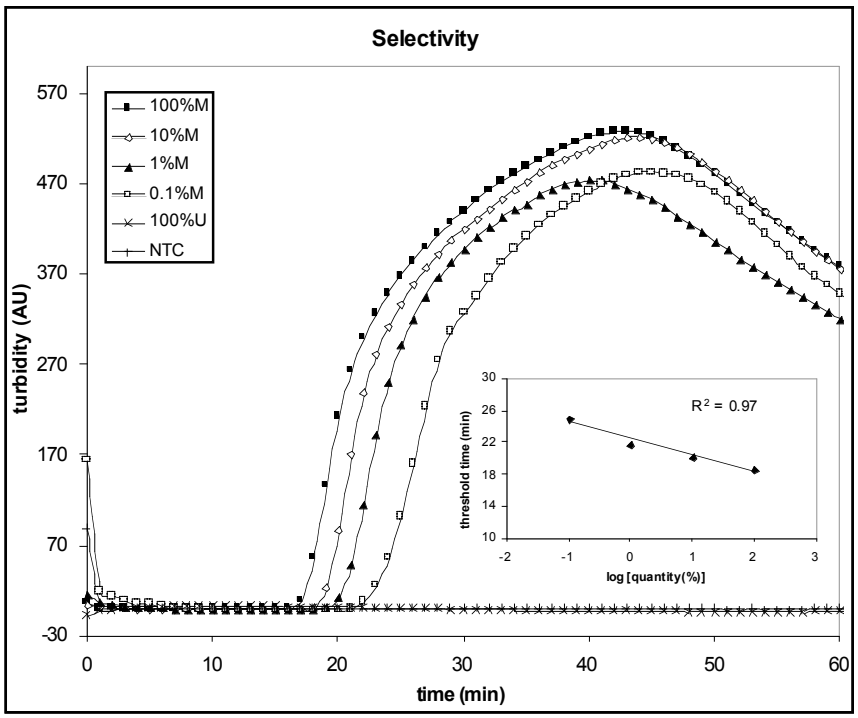


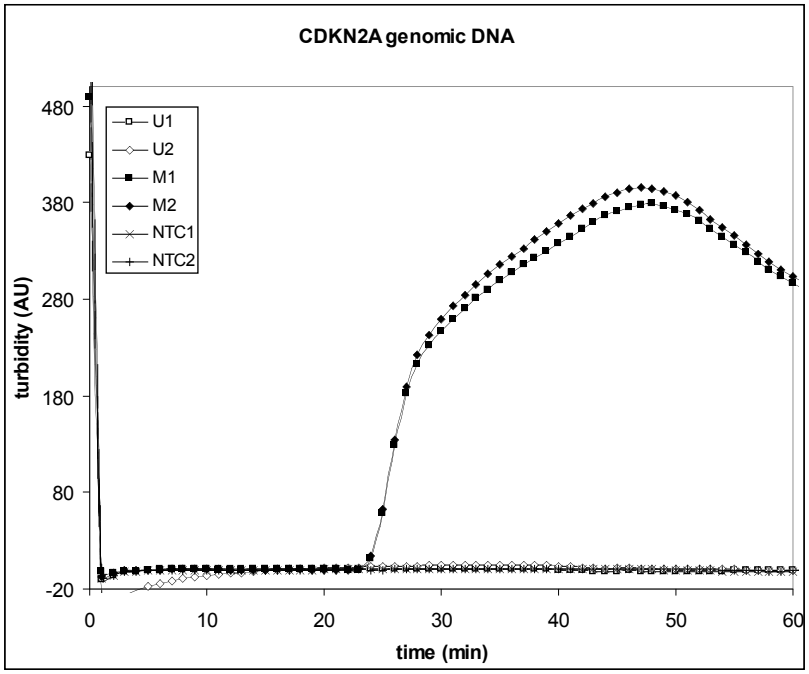
Fig. 25 Validation of MS-LAMP on synthetic plasmids: selectivity. Reactions contained decreasing amounts of DAPK methylated plasmid in a background of DAPK unmethylated plasmid. Methylated plasmid was detected down to 0.1% M/U with good linearity (R^2=0.97, *inset*).

Detection of CpG methylation on bisulfite treated genomic DNA controls

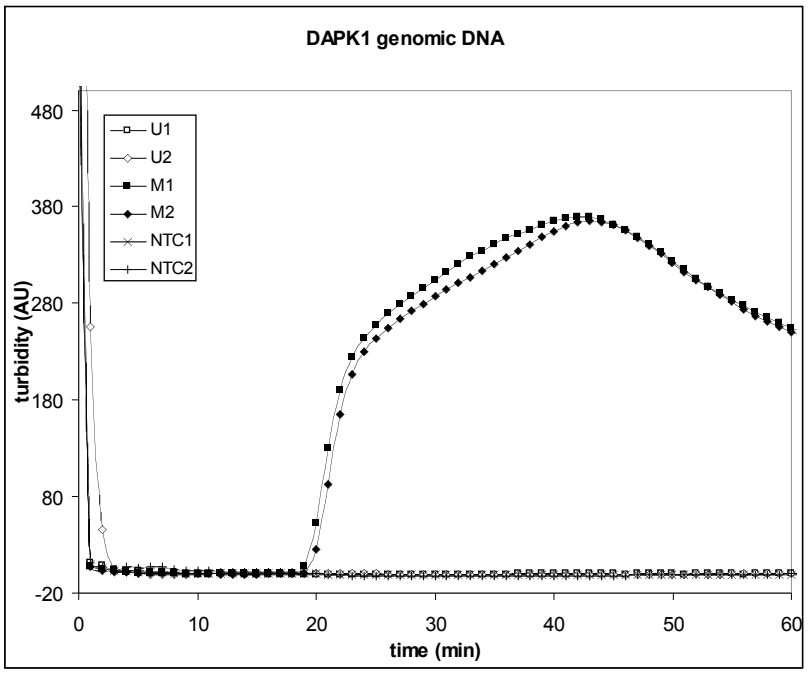
After the preliminary proof of principle of the performance of MS-LAMP on methylated and unmethylated targets synthetically obtained with no need for a bisulfite treatment, we set up a series of experiments to evaluate our technology in more realistic conditions. We purchased a commercially available genomic DNA either in fully methylated or unmethylated form. Unmethylated genomic DNA is a genomic DNA guaranteed as fully unmethylated (for example DNA from a fetal cell line) while the methylated version is pre-treated by the provider with DNA methyltransferases that link a methyl-group on every single CpG dinucleotide. Both samples were treated with sodium bisulfite (EZ-DNA kit) following the manufacturer's protocol to convert all the unmethylated cytosines into uraciles: final concentration of eluted DNA was 25 ng/μL .These DNA controls represent a very reliable control to test the performance of MS-LAMP and were used to assess the specificity and selectivity of MS-LAMP for the promoters of DAPK, CDKN2A and GATA5 on a bisulfite treated DNA.

Specificity

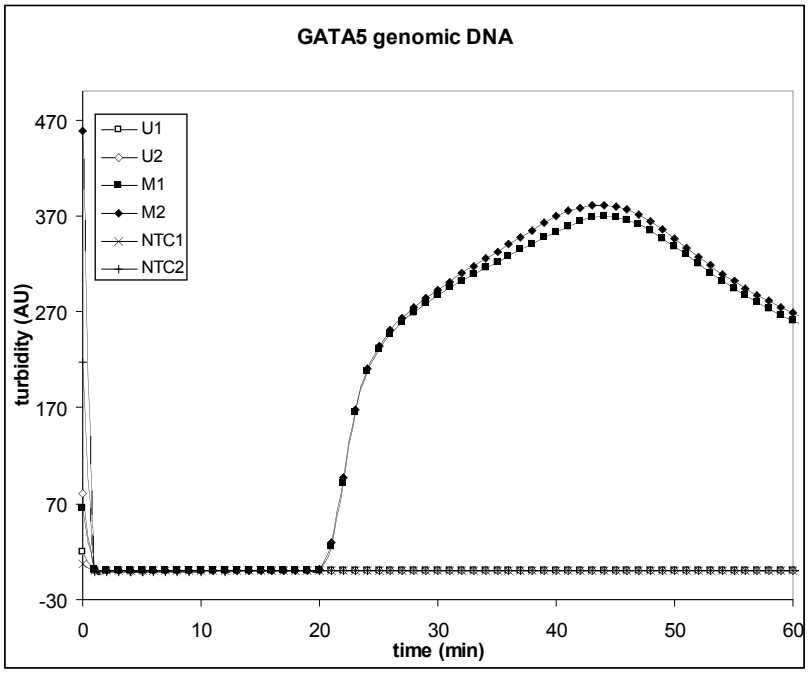
To assess specificity, we performed separate MS-LAMP reactions with the three assays using 50 ng of treated genomic DNA (either methylated and unmethylated) per reaction as target .
Results of amplification reactions are shown in **Figure 26**; no amplification occurred from unmethylated target and NTC while methylated targets produced an amplification within 30 minutes. Therefore MS-LAMP was able to specifically and rapidly detect and amplify the correct target DNA after a bisulfite treatment for each assayed promoter.


CDKN2A genomic DNA
U1
U2
M1
M2
NTC1
NTC2
turbidity (AU)
480
380
280
180
80
-20
0
10
20
30
40
50
60
time (min)

A


DAPK1 genomic DNA
U1
U2
M1
M2
NTC1
NTC2
turbidity (AU)
480
380
280
180
80
-20
0
10
20
30
40
50
60
time (min)

B



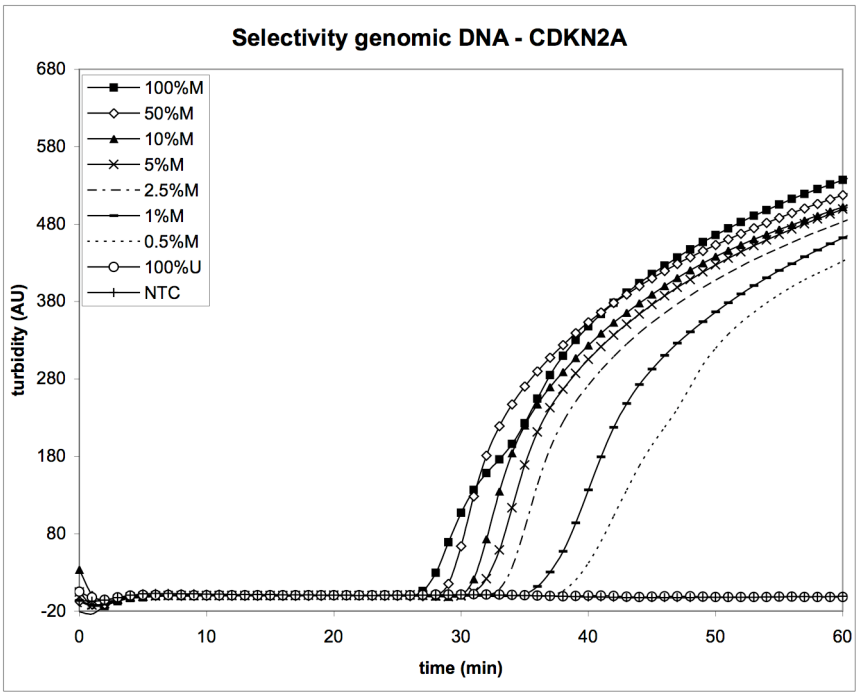
C

Fig. 26 MS-LAMP assays on genomic DNA controls: specificity. MS-LAMP assays were tested on 2 μl of commercial human genomic DNA either fully methylated (M) and unmethylated (U) after the bisulfite treatment. For the three developed assays p16 (A), DAPK (B) and GATA (C) duplicate experiments are depicted: unmethylated genomic DNA cannot be amplified within one hour reaction.

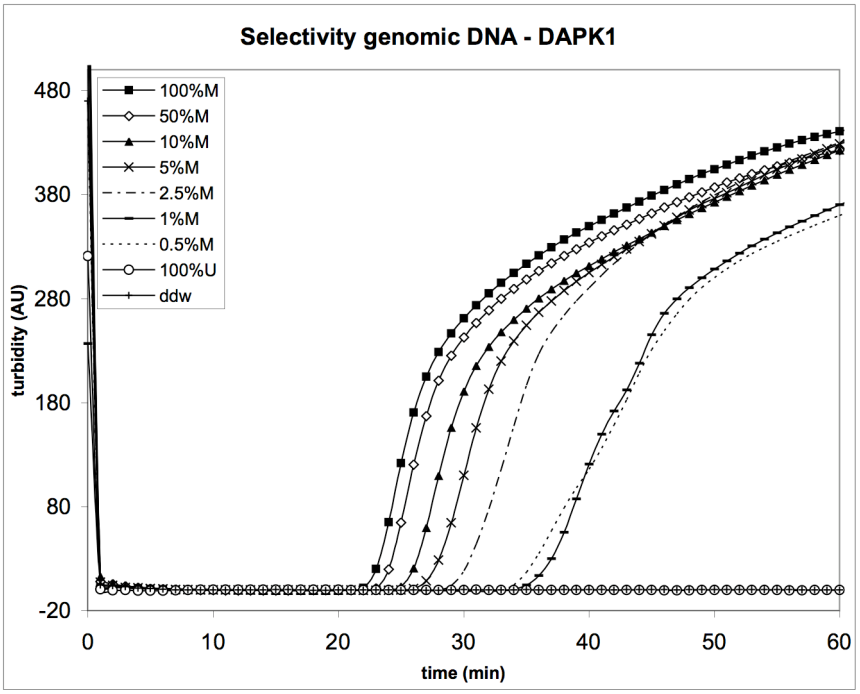
Selectivity

Once verified that all three MS-LAMP assays were able to discriminate between methylated and unmethylated DNA after a bisulfite treatment, we determined the lowest amount of hypermethylated genomic DNA that our assays were able to detect in a background of unmethylated DNA after a bisulfite treatment. We prepared human genomic DNA mixtures by serially diluting fully methylated DNA in fully unmethylated DNA to reach decreasing ratios from 100%M to 0.25%M; these mixtures were then treated with sodium bisulfite following the standard procedure and

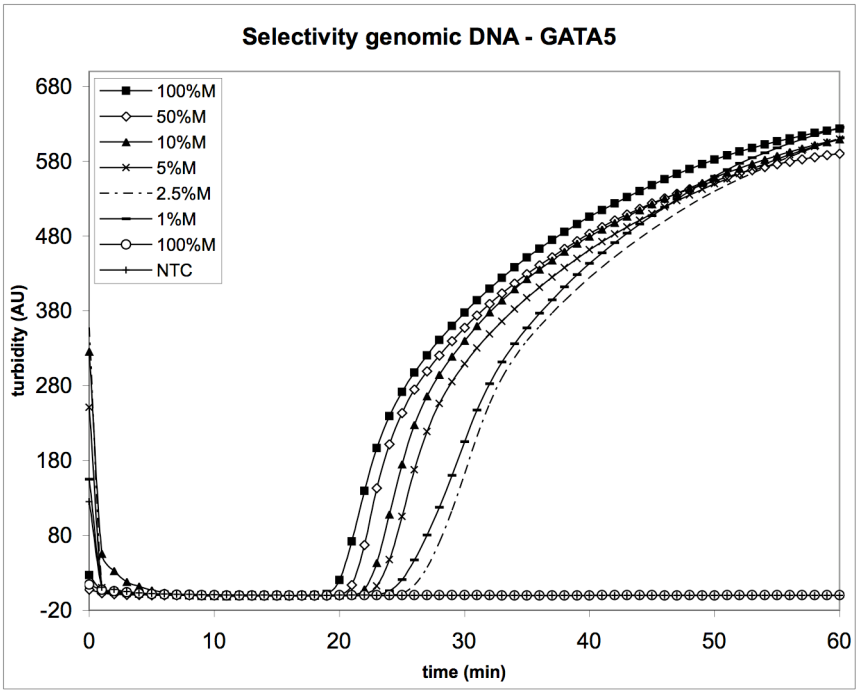
we assayed each resulting DNA preparation (50 ng per reaction) with the three assays. **Figure 27** shows amplification curves for each M/U ratio down to the lowest reproducible (3 repetitions) M/U ratio: we could detect methylated DNA down to 0.5%M for DAPK and p16 and 1%M for GATA5, meaning respectively 250 and 500 pg of methylated genomic DNA in an aspecific background (50 total ng DNA); a quantitative amplification pattern was also observed, even if a linear correlation between dose and response was not definable due to the narrow range of concentrations tested and to the not negligible phenomenon of DNA damage referable to bisulfite treatment. Specificity of the reaction was confirmed by NTC and fully unmethylated DNA reactions which remained negative for all the duration of the reactions.


Selectivity genomic DNA - CDKN2A
680
580
480
380
280
180
80
-20
turbidity (AU)
0
10
20
30
40
50
60
time (min)
100%M
50%M
10%M
5%M
2.5%M
1%M
0.5%M
100%U
NTC

A


Selectivity genomic DNA - DAPK1
480
380
280
180
80
-20
turbidity (AU)
0
10
20
30
40
50
60
time (min)
100%M
50%M
10%M
5%M
2.5%M
1%M
0.5%M
100%U
ddw

B

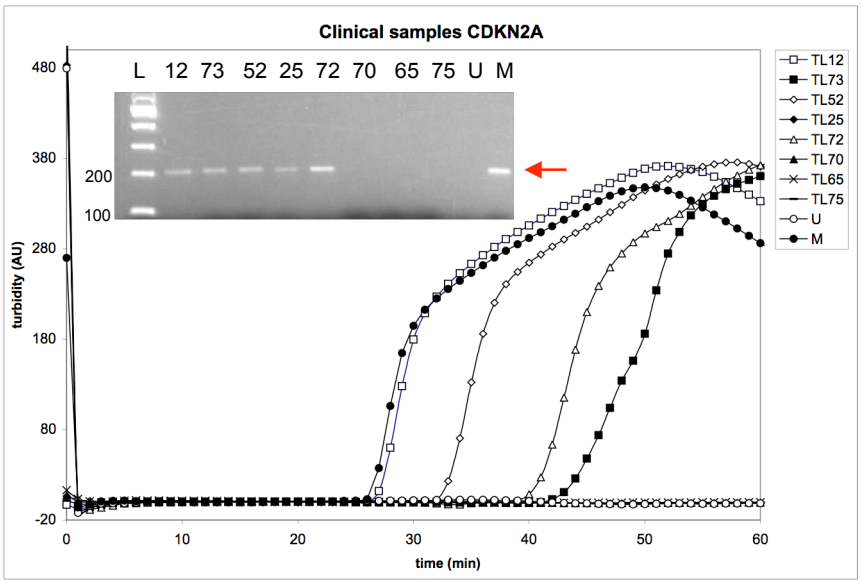


C

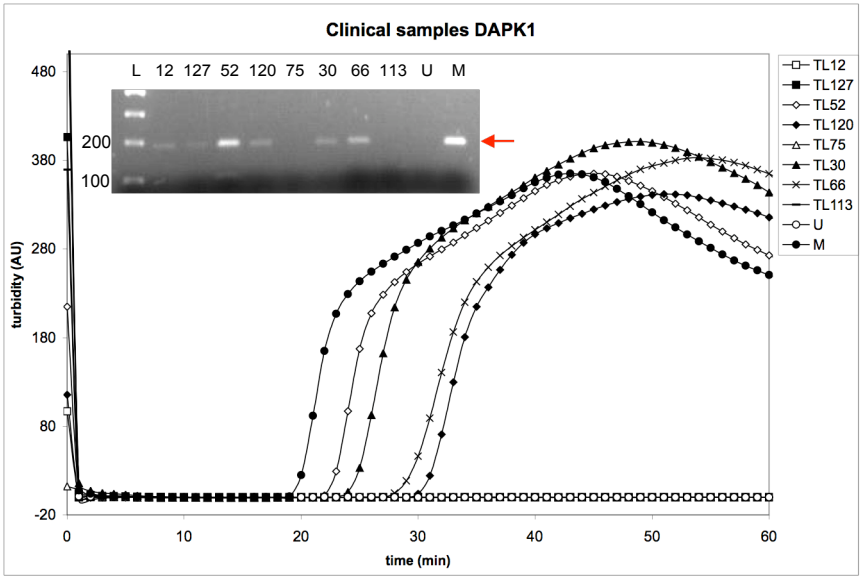
Fig. 27 MS-LAMP assays on genomic DNA controls: selectivity. P16 (A), DAPK (B) and GATA (C) assays were tested on mixes of decreasing concentrations of fully methylated genomic DNA diluted in a background of unmethylated genomic DNA and then treated with sodium bisulfite. 2 μl of each mix were assayed each time, containing a total DNA amount of 50 ng per reaction (approx. 15000 copies). Graph shows representative curves for each M/U ratio always detectable on a 3 repetitions basis. 0.5% M/U could be amplified for both p16 and DAPK and 1% for GATA.

Comparison between MS-LAMP and MS-PCR assays on clinical tumor samples

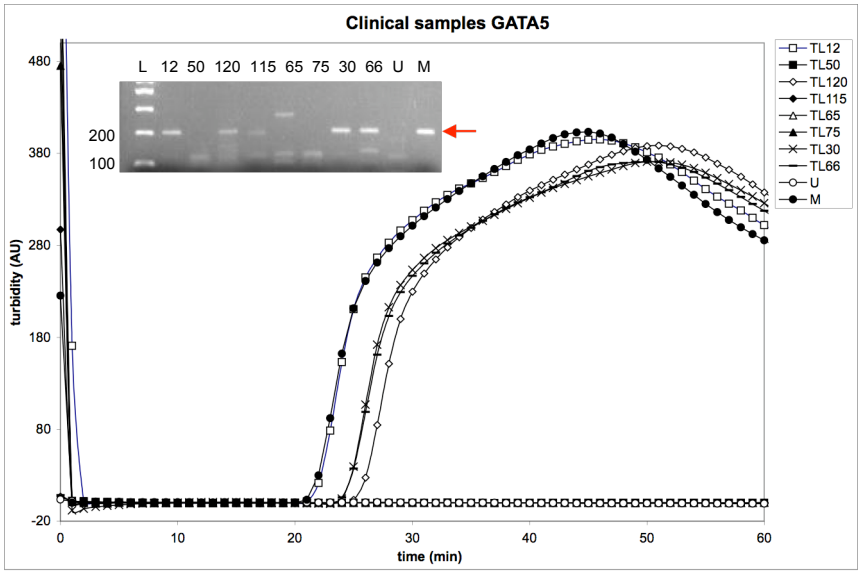
After the first phase in which we evaluated the specificity and the performance of our assays on plasmid and genomic controls we applied MS-LAMP for detecting the hypermethylation status in real clinical samples in comparison with an established method, methylation specific PCR (MS-PCR). We assayed the DNA methylation status of the three promoters (p16, DAPK and GATA5) in a total of 18 lung adenocarcinoma clinical specimens in parallel by MS-LAMP and MS-PCR after having performed the bisulfite treatment following the usual procedure. In order to assay a comparable DNA region with both the methods MS-PCR was performed using LAMP F3 and B3 as PCR primers. **Figure 28** shows amplification curves (not differing from those obtained from controls) of MS-LAMP reactions and electrophoretic gel runs after MS-PCR reactions for some representative samples; predicted amplicon lengths of MS-PCR are 192 bp (DAPK), 204 bp (p16) and 202 bp (GATA5).



A



B



C

Fig. 28 Clinical samples. Methylation assessment of a representative number of clinical samples and genomic controls by MS-LAMP and MS-PCR (inset) with the three assays (p16, A; DAPK, B, GATA, C). Red arrows indicate the expected bands.

Tables 2-4 summarize and compare in a contingency table form the results of the two analyses for the 18 samples: 8 samples were scored as methylation-positive by MS-LAMP for DAPK (44%), 9 for p16 (50%) and 13 for GATA (72%). Comprehensively we found a percentage of approximately 19% of discordant samples. All samples, except one, that were found methylation-positive for MS-LAMP were also scored as positive by MS-PCR. There were 9 cases out of 38 found methylation-positive via MS-PCR, that were negative via MS-LAMP.

DAPK

	positive for MS-PCR	negative for MS-PCR	
positive for LAMP	8	0	8
negative for LAMP	4	6	10
	12	6	

2

p16

	positive for MS-PCR	negative for MS-PCR	
positive for LAMP	9	0	9
negative for LAMP	4	5	9
	13	5	

3

GATA

	positive for MS-PCR	negative for MS-PCR	
positive for LAMP	12	1	13
negative for LAMP	1	4	5
	13	5	

4

Tables 2-4 Summary of the results obtained on lung tumor clinical samples by MS-LAMP and MS-PCR. Discordances are indicated in red.

One possible explanation of the observed lower sensitivity of MS-LAMP compared to MS-PCR can be the partial methylation of the target DNA, especially in the regions where LAMP primers anneal, that will prevent primer hybridization and consequent target amplification. To investigate this possibility, we decided to perform melting curve analysis of some representative MS-PCR amplification products of methylated samples, in presence of an intercalating dye. A partially methylated target will have a lower CG content after bisulfite conversion compared to a fully methylated target, because methylated cytosines are protected from conversion to thymines. The lower CG content will result in a lower melting temperature. As a example **Figure 29** shows the GATA amplicon melting curves for sample TL12, TL30 and TL66. A fully methylated control was also included. Sample TL66 shows a melting peak almost overlapped to that of the methylated control, while samples TL12 and TL30 show amplicon melting peaks at lower temperatures.
To estimate the expected difference in melting temperature between a fully methylated and a partially methylated amplicon we used Visual OMP (DNA Software Inc, USA), an advanced software for nucleic acid thermodynamics calculation. Given the experimental conditions (concentration, ionic strength, glycerol, denaturants etc…), this *in silico* tool can perform very precise simulations of the thermodynamic behavior of nucleic acids in aqueous solutions. We calculated the Tm of the amplicons produced by GATA MS-PCR reactions in a fully methylated version or by substituting thymines to 3, 5 or 8 predicted cytosines to simulate a possible partially methylated promoter (**Table 5**). The results of both the methylated control and of the simulated partially methylated sample agreed with the experimental evidences, showing a concordance between the experimental melting peak of the hypermethylated amplicon and its *in silico* predicted Tm and moreover a detectable and progressive Tm decrease by few C→T substitutions (3, 5 or 8 on a total of 24 cytosines in a 202 bp amplicon), strengthening our hypothesis on partial methylation.

To further confirm this hypothesis we looked at the real nucleotide sequence of these amplicons. Dye-terminator sequencing results reported in **Figure 30** confirmed the fully methylated status of sample TL66 which has a sequence identical to the methylated control. On contrary, in samples TL12 and TL30 some sequence positions expected to contain cytosines from the hypermethylated control were found to contain thymines or at least presented a double peak (cytosine and thymine) in the chromatogram. These findings indicate that some samples identified as methylated by MS-PCR where indeed only partially methylated. Similar results were observed testing by the same procedures DAPK and p16 promoters (data not shown).

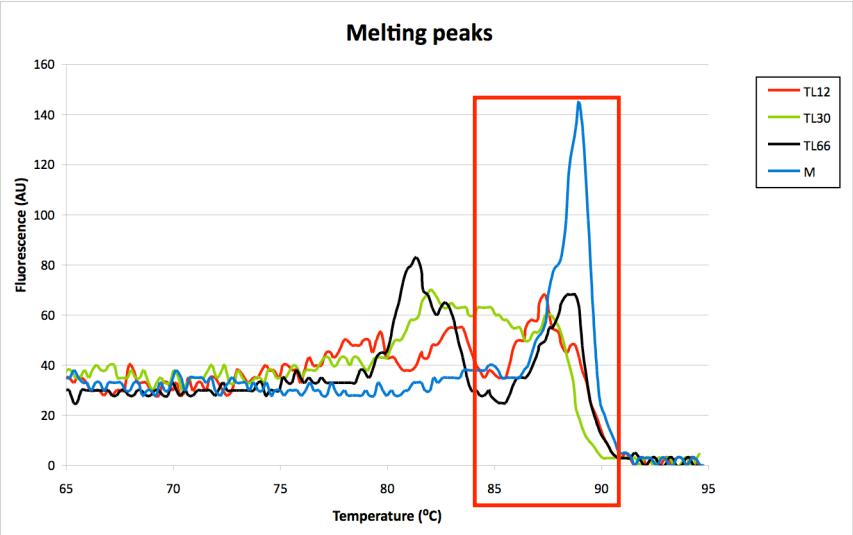


Fig. 29 Melting analysis of GATA MS-PCR amplification products. Melting curves of three representative samples and a hypermethylated genomic control obtained in a real-time PCR instrument by LC-green intercalating dye. Red square highlights the area where the peak of the GATA amplicon can be identified.

	# C>T	Species	Structure	ΔG°	ΔH	ΔS	Tm
methylated + methylated_rc	/	HETERODIMER	OPTIMAL	-118,24	-1684,1	-4630,7	87,9
partially_methylated + partially_methylated_rc	3	HETERODIMER	OPTIMAL	-115,15	-1675,1	-4613,2	87,4
partially_methylated2 + partially_methylated2_rc	5	HETERODIMER	OPTIMAL	-113,16	-1669,4	-4602,2	87
partially_methylated3 + partially_methylated3_rc	8	HETERODIMER	OPTIMAL	-109,83	-1660,2	-4584,9	86,3

Table 5 Results of the simulation by Visual OMP software. To verify the reliability of melting peaks, Tm of a fully methylated and differently partially methylated (3, 5 and 8 C>T substitutions) GATA amplicons were simulated specifying the experimental conditions used in the MS-PCR.

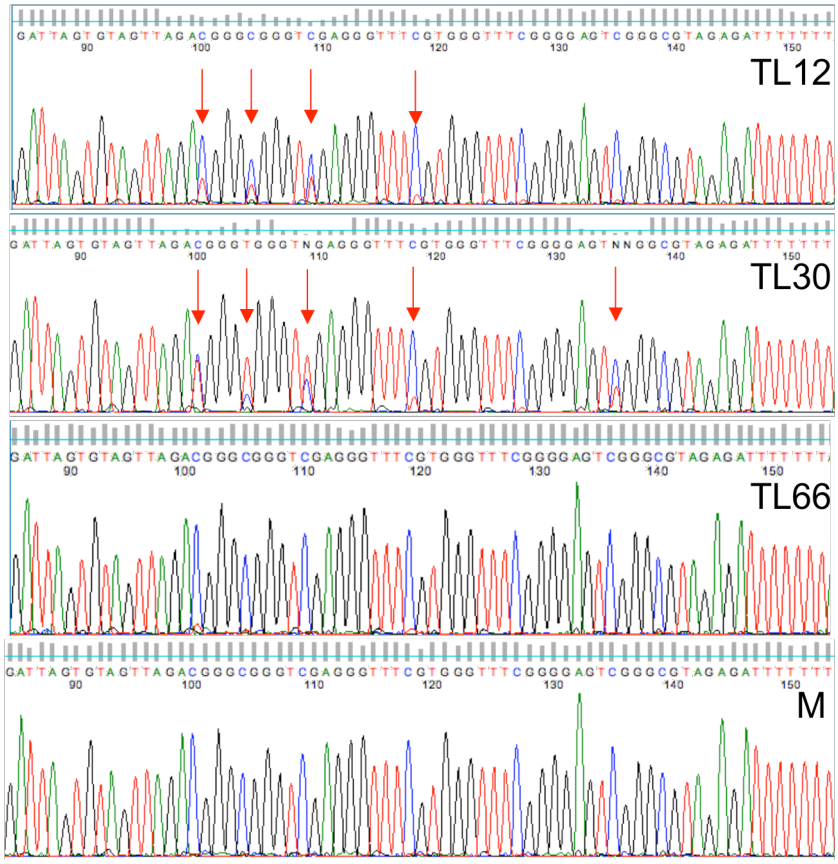


Fig. 30 Sequencing of three GATA MS-PCR amplification products. Chromatograms from dye-terminator sequencing of three representative clinical samples and a genomic fully methylated control MS-PCR amplicon. Arrows indicate some positions in the amplified DNA strands where predicted thymines have been replaced by cytosines (partial methylation) or where both nucleotides could be found. The amplification product of a control hypermethylated genomic DNA was also sequenced.

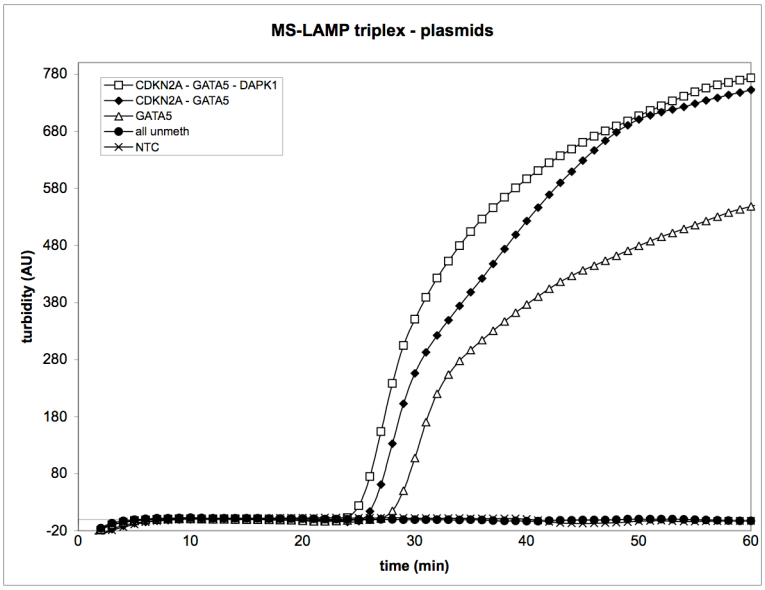
Multiplex MS-LAMP

One of MS-LAMP's major characteristics is its high specificity which can be explained by two complementary reasons: the high number of target recognition events and the lower risk of amplifying primer dimers, due to the peculiar isothermal amplification which requires a specific self-annealing mechanism with formation of loops which can occur only by a primer-target interaction. We exploited this key feature of the technology to develop a multiplex assay able to detect the hypermethylation status of anyone of the three promoters of interest assayed in parallel.
This multiplex application was developed by two different detection approaches: turbidimetry and fluorescence.

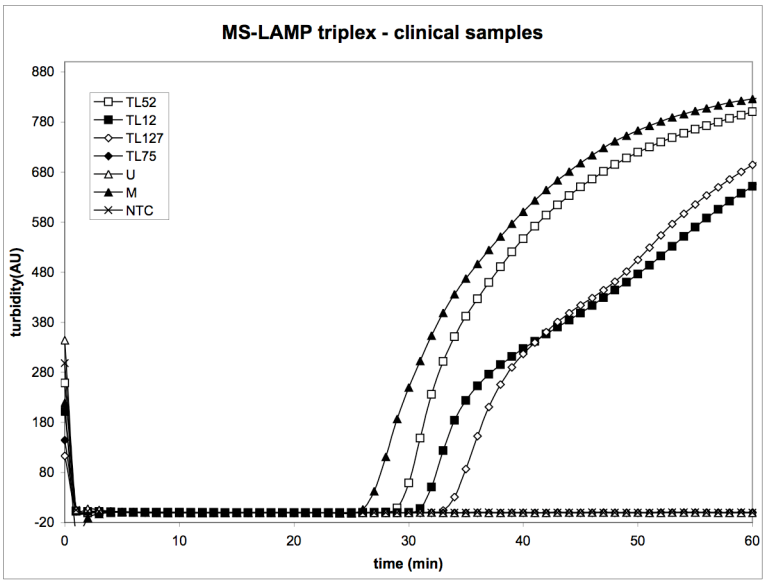
Turbidimetry multiplex reaction

All 18 primers constituting the three LAMP assays were included in a single reaction and this triplex assay was initially used to assay synthetic plasmid targets of the three promoters at various combinations (**Figure 31**, panel A). DNA amplification occurred either with a single (GATA) or two (GATA and p16) methylated plasmids in presence of the unmethylated counterparts of the remaining promoters and also when all the three methylated synthetic genes were mixed. No amplification was obtained in the reactions set up as negative controls containing only the unmethylated versions of the three plasmids at the same time or in no-target controls (NTC): this result shows that even in absence of an amplifiable target, the 18 primers composing the triplex were not able to generate any aspecific reaction product.

In order to evaluate the capability of this triplex assay to assign the methylation status of at least one of the selected promoter on a real genomic DNA, we assayed fully methylated and unmethylated genomic DNA controls together with four clinical samples (TL52, 12, 127 and 75) after a bisulfite treatment; the clinical specimens included in this preliminary analysis were found respectively with all three, two, one and no hypermethylated promoters in simplex MS-LAMP reactions. **Figure 31**, panel B, summarizing 3 independent experiments, shows no DNA amplification in NTC, in fully unmethylated genomic control and in the triple unmethylated clinical sample (TL75) while it confirms the hypermethylation status of fully methylated genomic control and of the three differently methylated clinical samples.



A



B

Fig. 31 Turbidimetry MS-LAMP triplex. Triplex reaction tested on different mixes contaning one, two or all three methylated plasmids and all three unmethylated plasmids (A). Triplex reaction in turbidimetry on clinical samples previously assayed in simplex format for the methylation state of the promoters (B). General methylation status of the plasmids and of the promoters was confirmed in triplex.

Fluorescent multiplex MS-LAMP

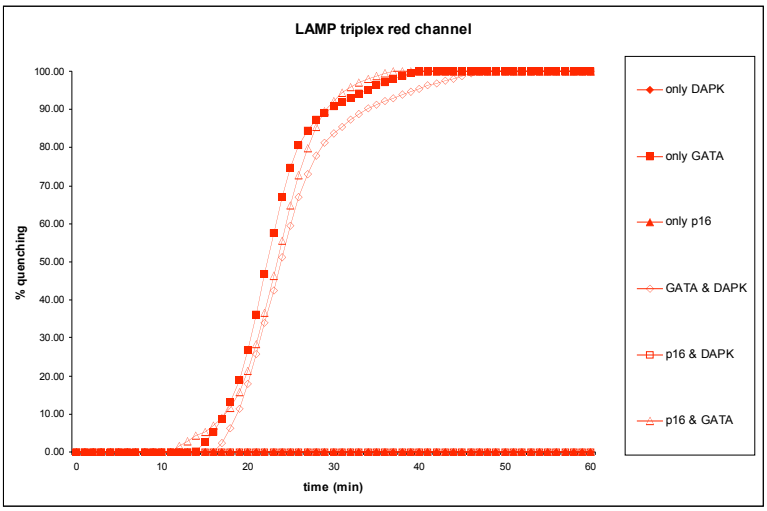
One of the latest developments of this work was also the evaluation of the capability of MS-LAMP to work in a multiplex application using fluorescence-based signal detection. While in the turbidimetry multiplex MS-LAMP reaction more than one amplification reactions occur at the same time but it is not possible to discriminate each single amplification event, fluorescent multiplex MS-LAMP should allow either many simultaneous amplifications and their individual recognition by using different fluorophores for different assays.
Although many established approaches were suitable for the generation and the detection of a fluorescent signal, we decided to apply and adapt a method known as guanine-quenching. Its principle is based on the quenching of a fluorescent dye when this fluorophore, being linked to 5' end of an oligonucleotide which anneals on its target and participates to an amplification reaction, stands in proximity of one or more guanosinic nucleotides. Thus the fluorescence signal is at its maximum before the beginning of amplification, decreases abruptly during the amplification and then reaches a stable plateau until the end of the reaction. This choice was dictated by the need of excluding a *hydrolysis probe* method like TaqMan, which relies on an exonucleasic activity that is lacking in the *Bst* polymerase. This suggested the use of *hybridization probe* method and guanine-quenching of a fluorophore resulted as particularly suitable due to the high efficiency of LAMP reaction which eventually results in an almost complete fluorophore quenching without the need for a further quencher dye. This makes this detection strategy also interestingly cost effective.

Differently labeled fluorescent primers were substituted to one of the two loop primers of each assay (see Materials and Methods) and the triplex reaction was then run in isothermal conditions in a real-time thermocycler and monitored simultaneously in the green, yellow and red channels.

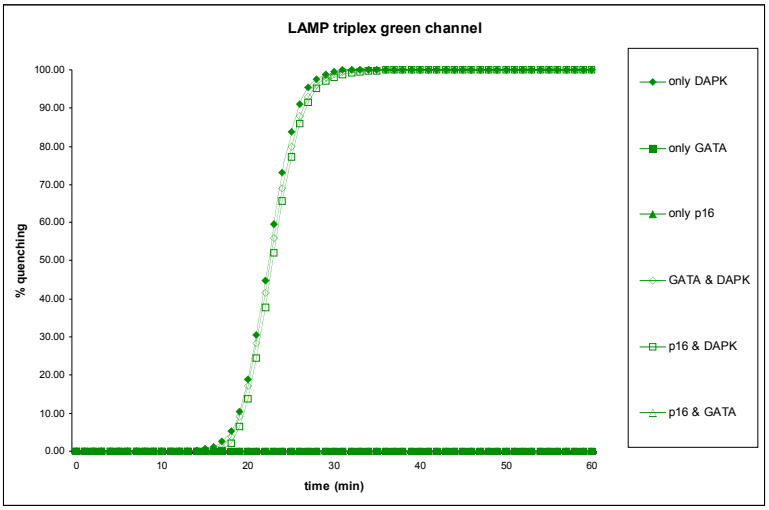
Comparing to turbidimetry reactions, the ability to discriminate the individual amplification reactions in the fluorescent multiplex LAMP, prompted a new optimization process: this was requested by the simultaneous occurrence of three independent reactions, characterized by different efficiencies which needed to be balanced either to ensure that enough common reagents could be available for the three amplification events and to avoid that any amplification, being too efficient, could suffocate the others (only one or both). Among common reagents, only an increase of *Bst* polymerase concentration seemed to improve the performance of the multiplex, while dNTPs had no effect. Finally,

because the DAPK assay was the fastest and the most efficient, its primers set concentration was decreased in order to have a balanced triplex assay and to not allow the two faster assays to consume the common resources before the last one could regularly occur.

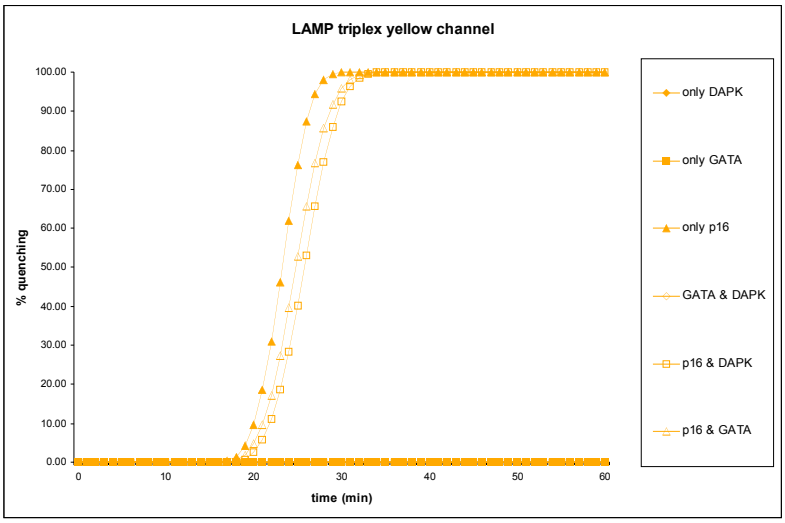
In order to test the level of specificity of our fluorescent multiplex assay, i.e. the capability of the assay to show the correct amplification signal depending on the provided target, different plasmids combinations were prepared to be employed as reaction targets (**Figure 32**): three mixtures contained separately each methylated plasmid in presence of the unmethylated form of the remaining two plasmids; three more mixtures contained each one of the possible combinations of two methylated plasmids together with the unmethylated form of the remaining one; finally two mixtures were constituted by all the three methylated or the three unmethylated controls at the same time. To further control the reliability of the assay we also tested the triplex MS-LAMP on both M and U genomic DNA controls. Each fluorescence channel showed a quenching of fluorescence of the corresponding dye only in the reaction tubes effectively expected to respond because containing the correct methylated target, either when this was the only methylated or when another methylated (amplifiable) target was present (panel A, B, C). No amplification curve could be detected in any fluorescence channel when only unmethylated versions of all the plasmids or fully unmethylated and treated genomic DNA were used as reaction targets (panel D), indicating that in the absence of a methylated and amplifiable target no aspecific reaction products are generated. In the triplex reaction where all the three methylated target were present at the same time (panel D), either in a simplified form (plasmids) or as fully methylated and treated genomic DNA, all three channels showed an amplification curve, indicating that all that three different reactions were taking place simultaneously in the same reaction tube, irrespective of the nature of the target DNA.


LAMP triplex red channel
% quenching
time (min)
only DAPK
only GATA
only p16
GATA & DAPK
p16 & DAPK
p16 & GATA

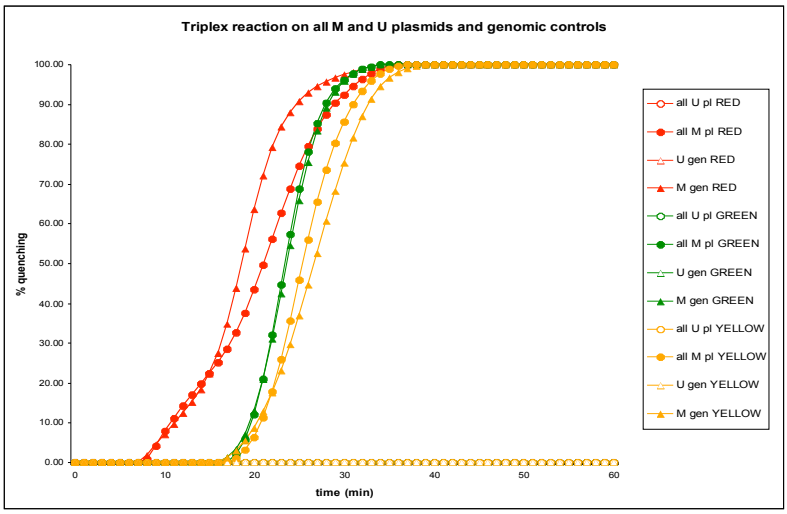
A


LAMP triplex green channel
% quenching
time (min)
only DAPK
only GATA
only p16
GATA & DAPK
p16 & DAPK
p16 & GATA

B



C



D

Fig. 32 Triplex MS-LAMP in fluorescence. Triplex reaction in fluorescence was monitored in the red, green and yellow channels of a real-time thermocycler to detect the amplification of respectively GATA, DAPK and p16 assays on plasmidic and genomic controls differently mixed. Panels A, B and C show respectively red, green and yellow channels results of LAMP reactions with either each single methylated plasmid or each combination of two methylated plasmids together. Panel D shows in the three colors the results in each channel on all three methylated or on all three unmethylated plasmids, on methylated or unmethylated genomic DNA. Curves are an elaboration of the original raw data (quenching curves) and are depicted as % of quenching against reaction time.

DISCUSSION

Aberrant methylation patterns of many promoters of human genes are increasingly recognized as related to the onset and to the behavior of many serious states of illness such as neuro-degenerative and autoimmune diseases and most importantly cancer[23]. Thus molecular detection of methylation status of promoters has an increasing relevance for understanding epigenetic mechanisms and for the correlation between methylation pattern of some promoters and onset and prognosis of many types of tumors[68,69,70].

LAMP is a DNA amplification technique characterized by several features which make it very interesting for molecular diagnostics applications in clinical routine: it is fast, since reactions are completed within 30 minutes; it is relatively inexpensive, not requiring complex instruments and allowing the use of inexpensive detection systems; it is comparable to PCR techniques in terms of sensitivity and quantitative performance. Moreover the most impressive feature of LAMP technique is the notable specificity, conferred by to the peculiar amplification mechanism, which allows to virtually eliminate primer dimers and aspecific amplifications[112].

During our design of LAMP primers sets we decided to position the 8 primer regions in order to obtain primers with very high specificity for methylated DNA after bisulfite conversion: thus we included as many CpG dinucleotides per primers as possible (average of 2-3 per primer region), compatibly with the design constraints. Further we designed our primers in a way that no CpGs were included at the extensible ends of any primer, within the last 3 nucleotides. By keeping the position of the CpG dinucleotides distant from the 3'-end of the primers, the effect of a single mismatch on priming efficiency is reduced. In this way, mismatches between the primer and one or more positions on the target do not abolish completely the priming efficiency but only reduce it. Thereby partial methylation, which is frequently encountered in cancerous tissues and which can impair DNA amplification in a regular PCR approach, can still lead to a detectable LAMP product, albeit having smaller yield than a fully methylated target that fits the primer exactly.

At the same time, target specificity (i.e. absence of mispriming at non-target regions) is maintained because LAMP operates only if all 6 primers bind to the target. Accordingly, by using 6 primers, LAMP

should be able to achieve both target specificity and quantitative amplification of partially methylated target regions. We then designed highly specific primer sets, obtaining a highly specific, sensitive and selective detection.

However, notwithstanding the rationale behind the design of LAMP primers we observed some discrepancies in the detection of possible hypermethylation patterns in clinical specimens when we compared our technique to MS-PCR. All these controversial cases except one were scored as negative results (unmethylated) from LAMP but were positive for MS-PCR. A possible reason for these unexpected results could be the high number of recognition events characterizing the LAMP assay resulting in a very high specificity which can negatively affect sensitivity in partially methylated samples: by probing 8 regions the primer set could become more sensitive to partial methylation in case of difficult samples even if no discrimination spots are included at the 3' of the primers. We found evidence of the partial methylation of our clinical samples, both by melting curve analysis of MS-PCR amplicons that showed melting peaks of some clinical samples at a slightly lower Tm comparing to methylated control and by sequencing, where chromatograms of those same samples presented thymine peaks instead of cytosines belonging to CpGs, foreseen from the published sequence and actually found when sequencing a methylated control. This indicates that some of the samples found positives by MS-PCR were only partially methylated. These samples resulted false negatives by MS-LAMP because of the too high specificity of our approach in which at least six different target regions are probed instead of only two regions probed in a PCR assay.
Nevertheless we also observed a single sample positive by GATA5 MS-LAMP and negative by GATA5 MS-PCR could be the result of the failure of MS-PCR on a partially methylated promoter.
For future developments we believe that reducing the number of discrimination points probed by LAMP primers, i.e. designing only some of the 8 primer regions on CpGs dinucleotides, while keeping the remaining primers in areas free from CpGs, could potentially decrease the risk of false negatives while keeping the assay specific.

The high specificity showed by MS-LAMP allows to integrate several simplex assays in a single reaction with a very limited risk of primer dimer formation, which can represent an issue in standard DNA amplification protocols for multiplex approaches and that could request time-consuming optimization processes.
Due to these features of MS-LAMP we could set up two different versions of the triplex assay. The first which was tested with success

both on controls and clinical specimens was obtained by mixing our three primers sets in a unique reaction in turbidimetry; the second (successfully tested, up to now, on plasmid controls) contained different oligonucleotides labeled with different fluorophores acting as loop primers. These dyes are quenched during LAMP reaction and allow a real-time fluorescence detection of the DNA amplification. The first approach benefits from ease and low cost of a turbidimetric reaction and could be used for a preliminary screening of clinical specimens in order to identify the presence of at least one methylated target. A fluorescence detection instead, would allow a simultaneous test for multiple promoters.

In conclusion LAMP assay, which is established for virology and microbiology approaches, was successfully adapted here for methylation detection with encouraging performance comparing to reference methods. It showed specificity, sensitivity and selectivity on both plasmid targets and sodium bisulfite-treated genomic DNA, by detecting down to 0.5% of methylated DNA in a background of 50 ng unmethylated DNA. Up to three different methylated targets were then simultaneously detected in multiplex reactions, both in real-time turbidimetry and in real-time fluorescence, with the same specificity of the individual assays. Eventually, the MS-LAMP technology was successfully tested on bisulfite-treated clinical lung tumor specimens. The comparison with the MS-PCR reference method showed a fairly good correlation. The observed discrepancies were mainly related to a combination of both the partial methylated status of the tested samples and the extreme specificity of our method which is due to the high number of discriminating regions. A modified primer design strategy aiming to the reduction of discriminating regions should allow to overcome this issue in the future. This would confer to MS-LAMP a sensitivity comparable to that of current gold standard methods even on low quality and partially methylated clinical samples, while maintaining its proven advantage in terms of higher specificity, shorter overall assay time and instrumental flexibility.

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