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REVIEW

# Long interspersed nuclear element-1 hypomethylation in cancer: biology and clinical applications

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**Abstract** Epigenetic changes in long interspersed nuclear element-1s (LINE-1s or L1s) occur early during the process of carcinogenesis. A lower methylation level (hypomethylation) of LINE-1 is common in most cancers, and the methylation level is further decreased in more advanced cancers. Consequently, several previous studies have suggested the use of LINE-1 hypomethylation levels in cancer screening, risk assessment, tumor staging, and prognostic prediction. Epigenomic changes are complex, and global hypomethylation influences LINE-1s in a generalized fashion. However, the methylation levels of some loci are dependent on their locations. The consequences of LINE-1 hypomethylation are genomic instability and alteration of gene expression. There are several mechanisms that promote both of these consequences in *cis*. Therefore, the methylation levels of different sets of LINE-1s may represent certain phenotypes. Furthermore, the methylation levels of specific sets of LINE-1s may indicate carcinogenesis-dependent hypomethylation. LINE-1 methylation pattern analysis can classify LINE-1s into one of three classes based on the number of methylated CpG dinucleotides. These classes include hypermethylation, partial methylation, and hypomethylation. The number of partial and hypermethylated loci, but not hypomethylated LINE-1s, is different among normal cell types. Consequently, the number of hypomethylated loci is a more promising marker than methylation level in the detection of cancer DNA. Further genome-wide studies to measure the methylation level of each LINE-1 locus may improve PCR-based methylation analysis to allow for a more specific and sensitive detection of cancer DNA or for an analysis of certain cancer phenotypes.

**Keywords** Long interspersed nuclear element-1s · DNA methylation · Hypomethylation · Partial methylation · Cancer · LINE-1

Because of the retrotransposition events that have occurred during evolution, the human genome contains more than 500,000 long interspersed nuclear element-1 (LINE-1 or L1) copies (Lander et al. 2001). Most LINE-1s are truncated. More than 10,000 LINE-1s are longer than 4.5 kb and consist of a 5′ untranslated region (UTR), two open reading frames, and a 3′ UTR containing a polyadenylation signal (Penzkofer et al. 2005). The DNA methylation levels of LINE-1 5′ UTRs in cancer have been extensively evaluated for potential use as an epigenomic marker for cancer (Chalitchagorn et al. 2004). The mean level of LINE-1 methylation in most cancer types is lower than in normal cells (Table 1). The degree of LINE-1 hypomethylation increases in more advanced cancers (Table 2 and Electronic supplementary material (ESM) Table 1). The methylation of other interspersed repetitive sequences (IRSs), such as Alu elements and human endogenous retrovirus (HERV) sequences, has been evaluated to a lesser extent (Tables 1 and 2 and ESM Table 1). LINE-1 and other IRS methylation levels have the potential to be used as universal tumor markers for

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**Table 1** Interspersed repetitive sequence hypomethylation in cancer

| Type of cancer | Repeated sequence | Hypomethylation | Reference |
|---|---|---|---|
| Abdominal paragangrioma | LINE-1 | Yes | Geli et al. (2008) |
| Breast cancer | Alu | Yes | Cho et al. (2010) |
| | LINE-1 | Yes | Cho et al. (2010) |
| Cervical cancer | LINE-1 | Yes | Shuangshoti et al. (2007) |
| Cholangiocarcinoma | LINE-1 | Yes | Kim et al. (2009a) |
| Colorectal cancer | Alu | Yes | Kwon et al. (2010; Rodriguez et al. (2008) |
| | LINE-1 | Yes | Chalitchagorn et al. (2004; Suter et al. (2004); Matsuzaki et al. (2005); Estecio et al. (2007); Iacopetta et al. (2007); Ogino et al. (2008a); Nosho et al. (2009a, b); An et al. (2010); Baba et al. (2010); Ibrahim et al. (2011); Irahara et al. (2010); Kawakami et al. (2011); Kwon et al. (2010) |
| Ependymoma | Alu | Yes | Xie et al. (2010) |
| Esophagus cancer | LINE-1 | Yes | Chalitchagorn et al. (2004) |
| Gastric cancer | Alu | Yes | Yoo et al. (2008); Park et al. (2009); Hou et al. (2010); Xiang et al. (2010); Yoshida et al. (2011) |
| | LINE-1 | Yes | Chalitchagorn et al. (2004; Yoo et al. (2008); Park et al. (2009); Yoshida et al. (2011) |
| Germ cell tumor | LINE-1 | Yes | Alves et al. (1996) |
| Fibrolamellar carcinoma of liver | LINE-1 | No | Trankenschuh et al. (2010) |
| Head and neck squamous cell cancer | LINE-1 | Yes | Chalitchagorn et al. (2004); Hsiung et al. (2007); Smith et al. (2007); Subbalekha et al. (2009) |
| Hepatoma | Alu | Yes | Lee et al. (2009) |
| | LINE-1 | Yes | Takai et al. (2000); Chalitchagorn et al. (2004); Tangkijvanich et al. (2007); Lee et al. (2009); Kim et al. (2009b); Formeister et (al. 2010); Trankenschuh et al. (2010) |
| Leukemia (acute promyelocytic leukemia) | Alu Yb8 | No | Choi et al. (2009) |
| | LINE-1 | No | Choi et al. (2009) |
| Leukemia (chronic myelogenous leukemia) | Alu | Yes | Roman-Gomez et al. (2008); Fabris et al. (2011) |
| | LINE-1 | Yes | Roman-Gomez et al. (2008); Roman-Gomez et al. (2005); Fabris et al. (2011) |
| Leukemia (plasma cell leukemia) | LINE-1 | Yes | Bollati et al. (2009) |
| Lung cancer (non-small cell lung cancer) | Alu | Yes | Daskalos et al. (2009) |
| | LINE-1 | Yes | Chalitchagorn et al. (2004); Daskalos et al. (2009); Jin et al. (2009); Saito et al. (2010) |
| Lymphoma | LINE-1 | No | Chalitchagorn et al. (2004) |
| Malignant peripheral nerve sheath tumor | LINE-1 | No | Feber et al. (2011) |
| Melanoma | LINE-1 | Yes | Tellez et al. (2009) |
| Multiple myeloma | Alu | Yes | Bollati et al. (2009) |
| | LINE-1 | Yes | Bollati et al. (2009) |
| Neuroendocrine tumor | Alu | Yes | Choi et al. (2007) |
| | LINE-1 | Yes | Choi et al. (2007) |
| Neurofibromatosis | LINE-1 | No | Feber et al. (2011) |

**Table 1** (continued)

| Type of cancer | Repeated sequence | Hypomethylation | Reference |
|---|---|---|---|
| Ovarian cancer | AluHER | Yes | Watts et al. (2008) |
| | V-W | Yes | Menendez et al. (2004) |
| | LINE-1 | Yes | Menendez et al. (2004); Pattamadilok et al. (2008); Woloszynska-Read et al. (2008); Dammann et al. (2010) |
| Parathyroid adenoma | LINE-1 | No | Juhlin et al. (2010) |
| Pheochromocytoma | LINE-1 | Yes | Geli et al. (2008) |
| Prostate cancer | Alu | Yes | Kim et al. (2011) |
| | LINE-1 | Yes | Santourlidis et al. (1999; Schulz et al. (2002); Chalitchagorn et al. (2004); Florl et al. (2004); Kindich et al. (2006); Yegnasubramanian et al. (2008); Cho et al. (2009) |
| Renal cell carcinoma | LINE-1 | No | Florl et al. (1999); Chalitchagorn et al. (2004) |
| Thyroid cancer (follicular type) | LINE-1 | No | Lee et al. (2008) |
| Thyroid cancer (papillary type) | LINE-1 | No | Chalitchagorn et al. (2004) |
| Urothelial cancer | HERV-K | Yes | Florl et al. (1999) |
| | Alu Yb8 | Yes | Choi et al. (2009) |
| | LINE-1 | Yes | Jurgens et al. (1996); Florl et al. (1999); Neuhausen et al. (2006); Choi et al. (2009); Wilhelm et al. (2010); Wolff et al. (2010) |

the detection of cancer DNA and to predict prognosis (Watanabe and Maekawa 2010).

LINE-1s have often been referred to as parasitic or junk DNA sequences. However, many LINE-1s play a role in gene regulation, and this control is regulated by the 5′ UTR methylation level (Aporntewan et al. 2011). As a result, changes in the methylation status of different sets of LINE-1 loci may lead to different cellular phenotypes (Phokaew et al. 2008; Aporntewan et al. 2011). These differences may be an underlying reason why LINE-1 methylation levels in normal cells show so much variation (Chalitchagorn et al. 2004). Lower methylation levels can also be found in many non-malignant conditions. Current PCR-based techniques were designed to measure LINE-1 methylation level and cannot distinguish between malignant- and non-malignant-associated LINE-1 hypomethylation (Xiong and Laird 1997; Laird 2010; Weisenberger et al. 2005; Yang et al. 2004). Therefore, a technique that measures not only the level but also the pattern of LINE-1 methylation should improve detection specificity and sensitivity and broaden the applications of this tumor marker. The topics of this review therefore include the following: (1) an up-to-date review of studies on LINE-1 and other IRS methylation levels in cancer; (2) the characteristics of LINE-1 hypomethylation in cancer; (3) the locus-dependent roles of LINE-1 hypomethylation in cancer development; and (4) the improvement in cancer DNA identification by LINE-1 methylation classification.

## LINE-1 and other IRS methylation levels in cancer

The methylation levels of LINE-1s, Alu elements, and some types of HERVs have been studied (Table 1). LINE-1 is the IRS element that is most frequently studied, and its hypomethylation has been found in many cancers. In a few cancer types, including cancer of the kidney, thyroid, and lymph nodes; acute promyelocytic leukemia; malignant peripheral nerve sheath tumor; and parathyroid adenoma, LINE-1 hypomethylation had not been found (Table 1). LINE-1 hypomethylation is also found in premalignant lesions of the cervix (Shuangshoti et al. 2007), extrahepatic bile duct (Kim et al. 2009a), and stomach (Park et al. 2009). Unexpectedly, LINE-1 hypermethylation was observed in some lesions that possess a high potential for malignant transformation, including lesions associated with myelodysplastic syndrome (Romermann et al. 2007) and liver cirrhosis (Takai et al. 2000). Interestingly, LINE-1 hypermethylation is found in partial hydatidiform moles, whereas

LINE-1 hypomethylation is seen in triploid diandric embryos. Both lesions originate from dispermic fertilization of an oocyte, suggesting that LINE-1 hypermethylation in moles is directly linked to the neoplastic process and is not a consequence of growth control (Perrin et al. 2007).

As shown in Table 2 and ESM Table 1, LINE-1 hypomethylation is associated with advanced tumor stage, higher histological grade, and poor prognosis. LINE-1 hypomethylation increases with tumor size (Tangkijvanich et al. 2007) and with higher tumor stage (Florl et al. 1999; Kindich et al. 2006; Pattamadilok et al. 2008; Lee et al. 2009; Baba et al. 2010). With increasing histological grade, according to multistep carcinogenesis, LINE-1 hypomethylation levels are increased in many cancer types (Florl et al. 1999; Shuangshoti et al. 2007; Cho et al. 2007; Park et al. 2009; Iramaneerat et al. 2011; Pattamadilok et al. 2008). Furthermore, LINE-1 hypomethylation is correlated with chromosomal aberrations (Schulz et al. 2002; Cho et al. 2007; Choi et al. 2007; Ogino et al. 2008a; Bollati et al. 2009), the hypermethylation of tumor suppressor genes (Choi et al. 2007; Kim et al. 2009a), mutations of tumor suppressor genes (Iacopetta et al. 2007; Kim et al. 2009a), the alternate transcription of oncogenes (Wolff et al. 2010), and the deregulation of cancer genes (Woloszynska-Read et al. 2008). Therefore, LINE-1 hypomethylation is associated with malignant phenotypes in human cells, deregulating gene expression and accelerating DNA rearrangement. Interestingly, the LINE-1 hypomethylation level is inversely associated with microsatellite instability (Estecio et al. 2007; Iacopetta et al. 2007; Ogino et al. 2008a; Goel et al. 2010; Kawakami et al. 2011). This finding may indicate that microsatellite instability and LINE-1 hypomethylation are characteristics of different genomic instability mechanisms.

From a clinical point of view, LINE-1 hypomethylation is associated with tumor metastasis (Schulz et al. 2002; Choi et al. 2007), the recurrence rate (Formeister et al. 2010), and the mortality rate (Ogino et al. 2008b; Ahn et al. 2011). LINE-1 hypomethylation has been reported to be a prognostic marker in several types of cancer including the stage IA subgroup of non-small cell lung cancer (Saito et al. 2010), ovary (Pattamadilok et al. 2008), and colon (Ogino et al. 2008b; Baba et al. 2010). LINE-1 hypomethylation has been proposed to be used as a screening tool for cancer detection. LINE-1 hypomethylation is observed in blood leukocyte DNA (Hsiung et al. 2007; Wilhelm et al. 2010), serum (Chalitchagorn et al. 2004; Tangkijvanich et al. 2007), and oral rinse samples (Subbalekha et al. 2009). Moreover, LINE-1 hypomethylation has also been demonstrated to be a surrogate marker for predicting tumor treatment response and prognosis (Aparicio et al. 2009; Sonpavde et al. 2009; Bernstein et al. 2010; Fang et al. 2010; Kawakami et al. 2011).

Alu elements and HERV genomes have been studied less frequently (Table 1). Hypomethylation of Alu sequences was reported in nine cancers, whereas hypomethylation of HERV-K and HERV-W genomes was found in urothelial cancer (Florl et al. 1999) and ovarian cancer (Menendez et al. 2004), respectively. All of the Alu- and HERV-hypomethylated cancers also possess LINE-1 hypomethylation. Certain cancer phenotypes are associated with the methylation levels of certain IRS types. For example, HERV-K, but not LINE-1 and HERV-E, methylation levels are associated with poor prognosis and platinum resistance of ovarian clear cell carcinoma (Iramaneerat et al. 2011).

## Characteristics of LINE-1 and global hypomethylation in cancer

Transgenic mice with hereditary defects in DNA methyltransferase show increased risk of developing cancer (Gaudet et al. 2003). Therefore, global hypomethylation may be one of the mechanisms that promote carcinogenesis and is unlikely to be just a consequence of cancer development. However, lower genome-wide methylation levels have also been found in many conditions, such as embryogenesis (Migeon et al. 1991; Kremenskoy et al. 2003), aging (Lutz et al. 1972; Gonzalo 2010), congenital malformation (Wang et al. 2010), exposure to certain environments (Bollati et al. 2007), nutrition (Brunaud et al. 2003), and autoimmune diseases (Richardson et al. 1990). There is no report of increased cancer development risk in individuals with some of these conditions. Therefore, it is reasonable to hypothesize that the genomic distribution of IRS methylation levels is different in global hypomethylation-related conditions. Interestingly, in some conditions, the loss of genome-wide methylation is IRS type-specific. For example, hypomethylation of Alu elements and HERV-K, but not LINE-1, was found in aging cells (Jintaridth and Mutirangura 2011). However, LINE-1 hypomethylation has been demonstrated in many other conditions (Schulz et al. 2006). Because LINE-1 methylation levels can regulate host gene expression in *cis* (Aporntewan et al. 2011), it is reasonable to hypothesize that the reduction in LINE-1 methylation is the result of epigenomic heterogeneity. A simpler explanation is that even though two different cells possess the same number of LINE-1 loci and methylation levels, each LINE-1 locus may have a different level of LINE-1 methylation in these cells (Phokaew et al. 2008). Therefore, LINE-1 hypomethylation is a cancer biomarker that may be a diagnostic tool for many cancer types. However, LINE-1 hypomethylation is not specific to cancer. The inclusion of information regarding the genomic LINE-1 methylation distribution pattern should therefore be a promising way to improve and

**Table 2** Interspersed repetitive sequence hypomethylation and cellular, molecular phenotype

| Cancer | IRS | Cellular phenotype | | | Molecular association | Reference |
|---|---|---|---|---|---|---|
| | | Higher clinical stage | Poorer histological grade | Survival | | |
| Cervical cancer | L1 | NR | PE | NR | NR | Shuangshoti et al. (2007) |
| Cholangiocarcinoma | L1 | NR | PE | NR | PE for CIMP and TSG mutation | Kim et al. (2009a) |
| Colorectal cancer | L1 | NR | PE | NR | NR | Chalitchagorn et al. (2004) |
| | L1 | NR | NR | NR | PE for MSS | Matsuzaki et al. (2005) |
| | L1 | NR | NR | NR | PE for MSI and CIN | Deng et al. (2006) |
| | L1 | NR | NR | NR | NE for MSI | Estecio et al. (2007) |
| | L1 | PE | PE in mucinous histology | NR | NE for MSI and TSG mutation | Iacopetta et al. (2007) |
| | L1 | NR | NR | NR | NE for MSI and CIMP<br>PE for chromosomal alteration in non-MSI tumor | Ogino et al. (2008a) |
| | L1 | NR | NR | PE | NR | Ogino et al. (2008b) |
| | L1 | NR | NR | NR | NE for SNPSs in one-carbon pathway genes. | Hazra et al. (2010) |
| | L1 | NR | NR | NR | LINE-1 methylation level correlated between synchronous cancer pairs from the same individuals. | Nosho et al. (2009a) |
| | L1 | NR | NR | NR | NE for *DNMT3B*- positive tumors | Nosho et al. (2009b) |
| | L1 | NR | NR | NR | PE for CIMP | An et al. (2010) |
| | L1 | NR | NR | PE in proximal colon cancer NE in distal colon cancer | NR | Ahn et al. (2010) |
| | L1 | NR | NR | PE | PE for MSI, CIMP, CIN, TSG mutation and TSG expression | Baba et al. (2010) |
| | L1 | NR | NR | NR | NE for MSI and methylation index<br>PE for MSS HNPCC | Goel et al. (2010) |
| | L1 | NR | PE | NR | NR | Ibrahim et al. (2011) |
| | L1 | NR | NR | PE | NE for MSI and CIMP | Kawakami et al. (2011) |
| | Alu, L1 | NE | PE | NR | NR | Kwon et al. (2010) |
| Ependymoma | Alu | NR | PE | NR | NR | Xie et al. (2010) |
| Gastric cancer | Alu | NR | PE | NR | NR | Park et al. (2009) |
| | L1 | NR | PE | NR | NR | Park et al. (2009) |
| | L1 | NR | NR | NR | PE for folate metabolizing gene polymorphisms | Hou et al. (2010) |
| Gastrointestinal stromal cancer | L1 | PE | NR | NR | NR | Igarashi et al. (2010) |
| Head and neck cancer | L1 | PE | NR | NR | NR | Smith et al. (2007) |
| | L1 | NR | NR | PE especially HPV 16 negative SCC | NR | Furniss et al. (2008) |
| | L1 | NS | NS | NR | NR | Subbalekha et al. (2009) |

**Table 2** (continued)

| Cancer | IRS | Cellular phenotype | | | Molecular association | Reference |
|---|---|---|---|---|---|---|
| | | Higher clinical stage | Poorer histological grade | Survival | | |
| Hepatocellular carcinoma | L1 | PE | PE | NR | NR | Tangkijvanich et al. (2007) |
| | Alu | NE | PE | NR | NR | Lee et al. (2009) |
| | L1 | PE | PE | NR | NR | Lee et al. (2009) |
| | L1 | NR | PE | NR | NR | Kim et al. (2009b) |
| | L1 | NR | NR | PE | NR | Formeister et al. (2010) |
| Multiple myeloma (MM) | Alu | NR | PE | NR | NE for hyperdiploid MM | Bollati et al. (2009) |
| | L1 | NR | PE | NR | PE for chromosomal translocation | Bollati et al. (2009) |
| Nerve tumor | L1 | NR | NE | NR | NR | Feber et al. (2011) |
| Neuroendocrine tumor | Alu | NR | NE | PE | PE for TSG methylation | Choi et al. (2007) |
| | L1 | NR | NE | PE | PE for chromosomal alteration and gene methylation | Choi et al. (2007) |
| Non-small cell lung cancer (NSCLC) | L1 | NR | SCC > adenocarcinoma ($P<0.001$) | NR | NR | Jin et al. (2009) |
| | L1 | NR | NR | PE | NR | Saito et al. (2010) |
| Odontogenic tumor | L1 | NR | Ameloblastoma > KCOT ($P=0.001$) | NR | NR | Kitkumthorn and Mutirangura (2010) |
| Ovarian cancer | L1 | NS | NE | PE | NR | (Pattamadilok et al. 2008) |
| | L1 | NR | NR | NR | PE with TSG expression | Woloszynska-Read et al. (2008) |
| | L1 | NR | NR | NR | PE for follow-up patients treated with decitabine ($P<0.001$) | Fang et al. (2010) |
| | L1 | PE | NR | NR | PE for TSG methylation | Woloszynska-Read et al. (2011) |
| Ovarian clear cell carcinoma | L1 | PE | NR | NR | NR | Iramaneerat et al. (2011) |
| | HERV-E | PE | NR | NR | NR | Iramaneerat et al. (2011) |
| | HERV-K | PE | NR | PE | NR | Iramaneerat et al. (2011) |
| Pancreatic cancer | L1 | NR | NR | NR | PE for *MTHFR* polymorphisms | Matsubayashi et al. (2005) |
| Prostate cancer | L1 | PE | NR | NR | NR | Santourlidis et al. (1999) |
| | L1 | PE | NR | PE | PE with chromosomal aberration | Schulz et al. (2002) |
| | L1 | PE | NR | NR | NR | Kindich et al. (2006) |
| | Alu | PE | PE | NR | NR | Cho et al. (2007) |
| | L1 | PE | PE | NR | NR | Cho et al. (2007) |
| | L1 | NR | NR | PE | NR | Yegnasubramanian et al. (2008) |
| | L1 | NR | PE | NR | NR | Cho et al. (2009) |

**Table 2** (continued)

| Cancer | IRS | Cellular phenotype | | | Molecular association | Reference |
|---|---|---|---|---|---|---|
| | | Higher clinical stage | Poorer histological grade | Survival | | |
| Urothelial cancer | L1 | PE | PE | NR | NR | Florl et al. (1999) |
| | L1 | PE | PE | NE | NR | Neuhausen et al. (2006) |
| | L1 | NR | NR | NR | PE for *Met* oncogene alternate transcript | Wolff et al. (2010) |

*IRS* interspersed repetitive sequence, *L1* long interspersed nucleotide element-1, *NR* no report, *NS* non-significant, *PE* positive evidence, *NE* negative evidence, *TSG* tumor suppressor gene, *CIMP* CpG island methylator phenotype, *MSS* microsatellite stable, *MSI* microsatellite instability, *CIN* chromosomal instability, *SNP* single nucleotide polymorphism, *DNMT3B* DNA methyltransferase-3B, *HNPCC* hereditary nonpolyposis colorectal cancer, *MM* multiple myeloma, *HCC* hepatocellular carcinoma, *SCC* squamous cell carcinoma, *KCOT* keratocystic odontogenic tumor, *HERV-E* human endogenous retrovirus E, *HERV-K* human endogenous retrovirus K, *MTHFR* methylenetetrahydrofolate reductase

widen the applications of LINE-1 methylation as a tumor marker (Pobsook et al. 2011).

Although LINE-1 methylation levels are variable in both cancer and normal cells, the mechanisms that alter methylation levels may be different. Normal cells possess several patterns of LINE-1 methylation levels. The levels of some cell types are precise and limited to within a specific range. In other cases, such as in the esophagus and thyroid, the ranges are expanded (Chalitchagorn et al. 2004). Similar patterns can be observed when the methylation status of each LINE-1 locus is observed (Phokaew et al. 2008). Different loci possess different methylation levels. Some are limited in range and others have wider ranges. Levels of LINE-1 locus methylation between different cell types are usually different, but each locus reveals similar patterns regarding the range of methylation levels (Phokaew et al. 2008).

Comparison of methylation levels between LINE-1 loci in normal cells showed no significant correlation. This result suggests that the methylation level is locus-dependent (Fig. 1; Phokaew et al. 2008). In contrast, significant associations of methylation levels between LINE-1 loci were frequently found in cancer. Therefore, the mechanism causing LINE-1 hypomethylation in cancer occurs generally and in a genome-wide manner (Fig. 1; Phokaew et al. 2008). However, this mechanism may be biased toward some IRS sequences. Using microarray analysis, Szpakowski et al. (2009) reported that primate-specific LINE-1 elements and most of the younger, primate-specific retroelements were preferentially hypomethylated in samples of squamous cell carcinoma of the head and neck in comparison to non-tumor adjacent tissue and normal controls. The association of the methylation level between two LINE-1 loci was found to be highest if they were located in the same gene (Phokaew et al. 2008). Therefore, in addition to evolutionarily derived classifications, LINE-1 hypomethylation in cancer can be influenced by genomic location.

## LINE-1 methylation regulates gene expression in *cis*

The notion that LINE-1 is methylated to prevent the process of retrotransposition should be reevaluated. First, in the human genome, less than 100 LINE-1s are retrotransposition competent, and only a few LINE-1s have been shown to be responsible for retrotransposition events during human evolution (Sassaman et al. 1997). Although a recent study showed that LINE-1 retrotransposition may be common (Lupski 2010; Beck et al. 2010), this evidence fails to explain the methylation of the vast majority of retrotransposition-incompetent LINE-1s. The human genome possesses thousands of 5′ UTR-containing LINE-1s, and most of them are methylated to a certain degree (Chalitchagorn et al. 2004). It is unlikely that this

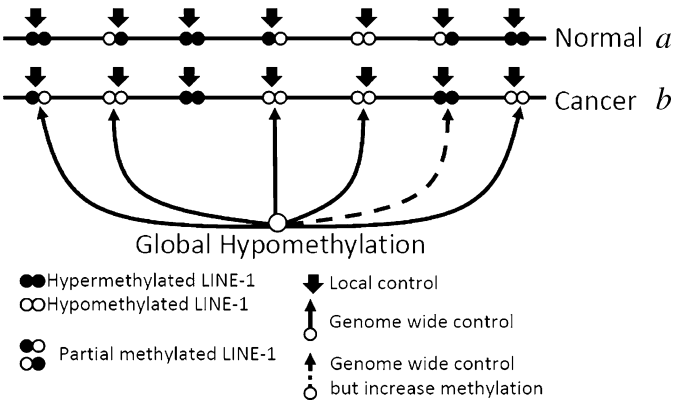


**Fig. 1** Effect of global hypomethylation in cancer. *a* Normal genomes contain hypermethylated, partially methylated, and hypomethylated LINE-1s. The methylation levels of each locus are regulated in a location-dependent manner. *b* The cancer genome contains more hypomethylated LINE-1s. Global hypomethylation decreases the methylation status of many LINE-1 loci. However, there are some loci that are not influenced and some loci that show increased methylation levels. Local mechanisms are also present in cancer cells, and some locations are affected by the process of carcinogenesis

methylation provides a selective advantage to the cells by preventing retrotransposition. The significant differences in LINE-1 methylation levels between loci or cell types suggests that LINE-1 methylation may be important to maintain normal cellular function and that this function may be altered by the global hypomethylation process that occurs in cancer.

The location-dependent LINE-1 methylation pattern in normal cells suggests a role for epigenetic regulation. Currently, there are at least two reported mechanisms for how LINE-1 methylation regulates gene expression in *cis*. Both mechanisms are dependent on the transcriptional activity of the LINE-1 promoter. Moreover, similar to other promoters, the LINE-1 5′ UTR promoter is controlled by DNA methylation, and the transcription activity of a LINE-1 element is directly correlated with its hypomethylation level (Aporntewan et al. 2011). The first mechanism is that LINE-1-mediated control of gene expression is through the production of unique RNA sequences (Fig. 2). The other mechanism is that intragenic LINE-1 RNAs repress host gene expression via the nuclear RNA-induced silencing complex (RISC; Fig. 3).

There are two ways for the LINE-1 promoter to produce unique RNA sequences (Fig. 2). The 5′ UTR of LINE-1 is a promoter that transcribes in both the forward and reverse directions (Matlik et al. 2006; Weber et al. 2010; Speek 2001; Wolff et al. 2010; Rangwala et al. 2009). If the transcription is in the forward orientation, then the promoter produces LINE-1 RNA. However, the poly-A addition signal of LINE-1 does not always function. Consequently, many LINE-1 transcripts can continue beyond the end of the LINE-1 sequence, therefore resulting in 3′ transduction (Moran et al. 1999; Rangwala et al. 2009). These transduction sequences are unique RNA sequences generated by the LINE-1 promoter. On the other hand, LINE-1 5′ transduction that occurs by reverse transcription will also produce unique RNA sequences. A large number of these transduction sequences have been reported (Rangwala et al. 2009); however, there are currently only two examples that prove that these sequences are increased by LINE-1 hypomethylation (Weber et al. 2010; Wolff et al. 2010; Aporntewan et al. 2011).

Intragenic LINE-1 regulation of host gene expression was revealed by the finding that in vitro insertion of a full-length LINE-1 disrupted host gene expression (Han et al. 2004). In vivo, this gene regulation is tuned by LINE-1 methylation levels (Aporntewan et al. 2011). When LINE-1 methylation levels were reduced by chemical treatment or by carcinogenesis, a significant number of genes containing LINE-1s were repressed (Fig. 3a–c). The degree of this repression was inversely correlated with the intragenic LINE-1 methylation level. The role of LINE-1 methylation is to prevent the formation of a pre-mRNA–LINE-1–RNA complex. If the complex is formed, then the RISC protein AGO2 will bind and prevent mRNA production (Fig. 3; Aporntewan et al. 2011).

Comparative sequence analysis between intragenic and intergenic LINE-1s showed multiple conserved nucleotides in intragenic LINE-1s that are crucial for maintaining LINE-1 transcription and methylation (Aporntewan et al. 2011). Moreover, many LINE-1s are excluded from genomic regions containing housekeeping genes (Eller et al. 2007; Graham and Boissinot 2006). Therefore, locations of LINE-1s yield a selective advantage for human evolution. It is important to note that the diploid human genome contains an extensive amount of structural variation due to retrotransposition events (Huang et al. 2010; Ewing and Kazazian 2011). Consequently, variation in the expression of many genes may be due to the distinctive locations of heritable LINE-1s, and similar to other DNA polymorphisms, some LINE-1 insertions are polymorphisms that lead to certain disease-related phenotypes. LINE-1 hypomethylation may also control gene expression in *trans*. In some cancer cells, inhibition of LINE-1 reverse transcriptase can alter the expression of many genes (Carlini et al. 2010).

## LINE-1 hypomethylation and genomic instability in cancer

In addition to a number of association studies (Ji et al. 1997; Lu and Randerath 1984; Daskalos et al. 2009), the high risk of chromosomal abnormalities in individuals with hereditary mutations in DNA methyltransferase genes indicates that global hypomethylation promotes genomic instability (Hansen et al. 1999; Eden et al. 2003). However, the underlying mechanisms of how DNA methylation

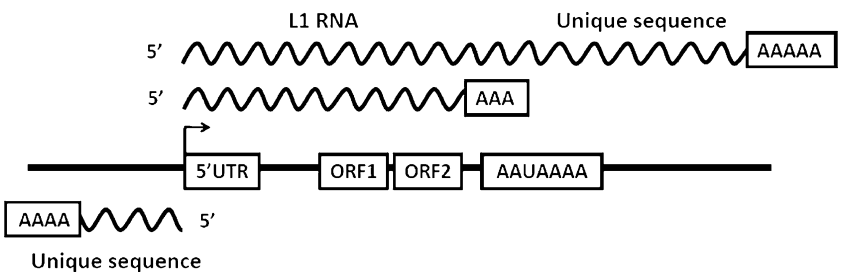


**Fig. 2** LINE-1 can produce two types of unique RNA sequences. One type of unique sequence is the result of LINE-1 RNA transcription proceeding beyond the LINE-1 sequence. The other type occurs when the reverse LINE-1 promoter transcribes unique DNA sequences located beyond the 5′ end of LINE-1

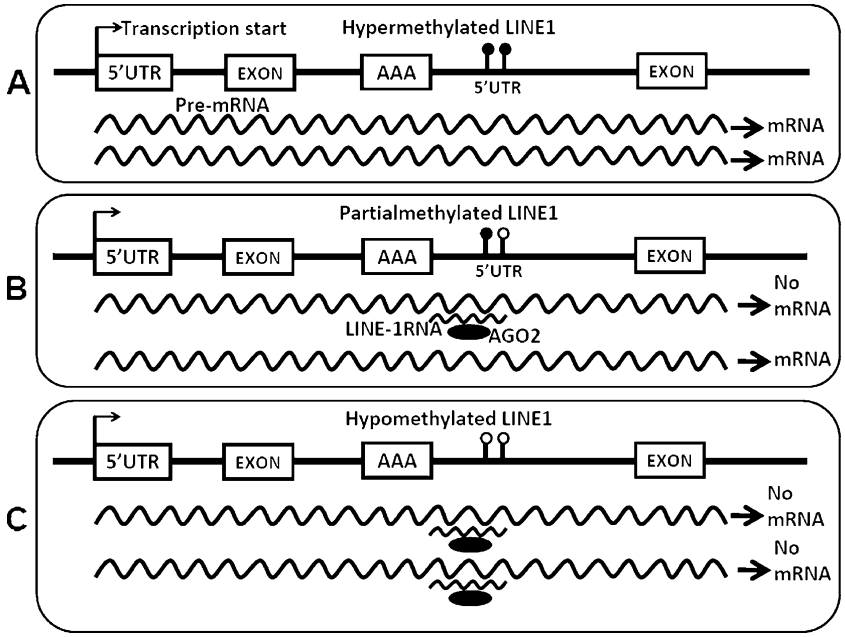


**Fig. 3** Intragenic hypomethylated LINE-1s repress host gene expression via AGO2. The schematic demonstrates that the same gene from three different cells has different levels of intragenic LINE-1 methylation. **a** Hypermethylated LINE-1. **b** Partially methylated LINE-1. **c** Hypomethylated LINE-1. LINE-1 RNA is produced when the methylation of the LINE-1 5′ UTR is reduced. The LINE-1 RNA–pre-mRNA complex is bound by AGO2, and mRNA production is prevented

maintains genomic integrity are not yet known. Current reports suggest that LINE-1 hypomethylation leads to several events that promote genomic instability, including retrotransposition, endogenous DNA double-strand break (EDSB) repair, and the dysregulation of DNA repair genes.

The process of LINE-1 retrotransposition includes RNA transcription, protein translation, DNA restriction, reverse transcription, and integration (Moran 1999). This retrotransposition usually produces large DNA rearrangements (Huang et al. 2010; Gilbert et al. 2002). Recently, an advanced LINE-1 junction sequencing technique showed that somatic L1 insertions occur at high frequency in human lung cancer genomes (Iskow et al. 2010). Therefore, LINE-1 hypomethylation in cancer may increase the retrotransposition activity of some LINE-1s and consequently cause a faster rate of DNA rearrangement. However, many DNA rearrangements occur in cancer cells that are not LINE-1 retrotransposition events. Therefore, LINE-1 retrotransposition contributes to only a small proportion of mutations in cancer. Moreover, there are only a few reports that retrotransposition events can produce clonal expansion mutations (Miki et al. 1992). Finally, the loss of the methylation of non-retrotransposable repeats, such as satellite DNA, also promotes chromosome translocation (Maraschio et al. 1988; Ji et al. 1997). Therefore, LINE-1 retrotransposition may not be the major mechanism causing somatic mutation in cancer by global hypomethylation.

The second mechanism is the differential repair of methylated and unmethylated replication-independent EDSBs (RIND-EDSBs; Kongruttanachok et al. 2010). RIND-EDSBs are different from replication-dependent EDSBs and environmental- or radiation-induced DSBs. Replication-dependent EDSBs and radiation-induced DSBs, if unrepaired, lead to cell death. In contrast, RIND-EDSBs are ubiquitously present in all cells and always involve hypermethylation (Pornthanakasem et al. 2008). This occurrence indicates a time lag between methylated RIND-EDSB production and repair (Kongruttanachok et al. 2010). RIND-EDSBs can be produced within both methylated and unmethylated genomes. Methylated RIND-EDSBs are selectively repaired by the more precise ataxia telangiectasia mutated (ATM)-dependent non-homologous end joining repair process (Kongruttanachok et al. 2010). Therefore, the RIND-EDSB repair process of hypomethylated genomes is faster and more error-prone. Because the LINE-1 methylation levels of each locus are distinct, the mutation rate caused by RIND-EDSB repair errors is dependent on the methylation status of the genome near the EDSBs. Currently, there are only two reports focused on RIND-EDSBs (Pornthanakasem et al. 2008; Kongruttanachok et al. 2010). Further studies are needed to explore the causes and roles of RIND-EDSBs and to determine how genomic hypomethylation promotes instability.

A third possible mechanism is that LINE-1 hypomethylation down-regulates DNA repair genes. One of these genes is *PPP2R2B*, which contains intragenic LINE-1s. In cancer, these LINE-1s are frequently hypomethylated and *PPP2R2B* is frequently down-regulated (Aporntewan et al. 2011). One of the functions of PPP2R2B is to increase nuclear ATM protein (Suyarnsestakorn et al. 2010). ATM is a serine/threonine protein kinase that is important in the activation of the DNA damage checkpoint, leading to cell cycle arrest, DNA repair, or apoptosis (Mavrou et al. 2008). A lack of ATM promotes genomic instability (Kim et al. 2002). Therefore, LINE-1 hypomethylation may indirectly promote genomic instability by interfering with ATM function.

## LINE-1 methylation patterns in normal and cancer cells

It is commonly assumed that LINE-1 elements in normal cells are completely methylated. Combined bisulfite restriction analysis or COBRA, deep sequencing, and microarray analysis demonstrated that the genomic distribution of the methylation of LINE-1s and other IRS loci is not homogenous (Phokaew et al. 2008; Xie et al. 2009, 2011; Szpakowski et al. 2009). The methylation levels of LINE-1 loci can be divided into three groups: hypermethylated, partially methylated, and hypomethylated (Pobsook et al. 2011). Classification is based on the number of methylated and unmethylated CpG dinucleotides (Fig. 1). In normal cells, the majorities of LINE-1 loci are hypermethylated or partially methylated. Few LINE-1 loci are hypomethylated. Comparisons between normal white blood cells and normal oral epithelium showed that even though LINE-1 methylation levels are different, the number of hypomethylated loci was not distinguishable between the two normal tissues (Fig. 4). Therefore, the differences in methylation levels between normal cell types are primarily influenced by the number of hypermethylated and partially methylated loci. In cancer cells, the methylation of a majority of LINE-1 loci is decreased, with some loci remaining unchanged and a few being increased when compared with normal cells. Thus, the number of hypomethylated loci is increased in cancer cells (Fig. 4).

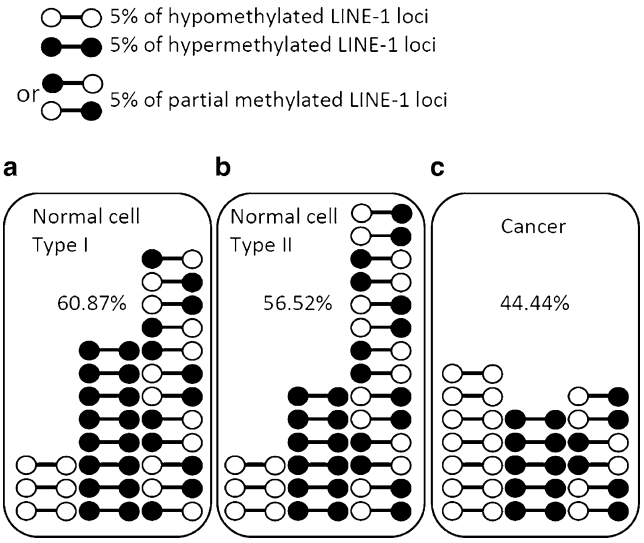


**Fig. 4** Examples of LINE-1 methylation patterns in three cells. The number of LINE-1 loci and the methylation levels were approximated from the average levels of a previous report (Pobsook et al. 2011). Type I normal cells (**a**), type II normal cells (**b**), and cancer cells (**c**) possess LINE-1 methylation levels of 60.87%, 56.52%, and 44.44%, respectively. Even though different normal cell types contain different methylation levels, the numbers of partially methylated, hypermethylated, and hypomethylated loci were not different. Cancer cells showed lower methylation levels and a lower number of partially methylated loci, but a higher number of hypomethylated LINE-1 loci

A recent report showed distinctive characteristics of LINE-1 partial methylation that was dependent on malignant transformation (Pobsook et al. 2011). In normal cells, the number of partially methylated LINE-1 loci in each sample was directly correlated with the number of hypomethylated loci, but was inversely associated with the number of hypermethylated loci. This result suggested that a dynamic form of LINE-1 epigenetic modification, between partial methylation and hypermethylation, is present in normal cells. Because hypomethylated LINE-1s were not distinguishable between different types of normal cells, the dynamic between the partially methylated and hypermethylated forms may be the cause of the variation in LINE-1 methylation levels between normal cell types. Moreover, the more partially methylated loci may represent the lower LINE-1 methylation level. In contrast, in the cancer genome, the number of partially methylated LINE-1s was directly correlated with the number of hypermethylated LINE-1s. Therefore, in striking contrast to the normal genome, partially methylated LINE-1 loci represent a subset of methylated LINE-1s in cancer cells (Pobsook et al. 2011). Current PCR-based techniques, by real-time quantitative PCR, COBRA, and pyrosequencing, determine LINE-1 hypomethylation levels by combining all unmethylated CpG nucleotides from both partially methylated or hypomethylated loci (Xiong and Laird 1997; Laird 2010; Weisenberger et al. 2005; Yang et al. 2004). Therefore, the sensitivity in distinguishing cancer DNA is low. Pobsook et al. (2011) also showed that excluding partial methylation loci from the count of hypomethylated LINE-1 loci improved the sensitivity and specificity of cancer DNA detection.

## From biology to clinical application and future direction of LINE-1 hypomethylation in cancer

Understanding how LINE-1 methylation levels change during multistep carcinogenesis has implications for diagnostic applications. Several LINE-1 and other IRS methylation studies have shown that global hypomethylation is a common epigenetic change in cancer (Table 1). Moreover, this process is directly correlated with cancer progression. Therefore, lower LINE-1 methylation levels have been shown to be associated with higher cancer stages and may also be a promising marker for the prognostic prediction of many cancers (Table 2 and ESM Table 1). Global methylation changes initiate early, and the genome becomes progressively hypomethylated during the process of multistep carcinogenesis. Therefore, LINE-1 and other IRS hypomethylation levels are candidate tumor markers for cancer (Table 2 and ESM Table 1).

There is a technical advantage to using PCR-based assays to measure IRS methylation levels. Multiple copies of IRSs are present in the genome; therefore, this detection method is highly sensitive even in poor-quality clinical DNA samples. These clinical samples include paraffin-embedded sections, plasma, and other fluid or washes, such as oral rinses (Chalitchagorn et al. 2004; Tangkijvanich et al. 2007; Aparicio et al. 2009; Subbalekha et al. 2009) (Vaissiere et al. 2009). LINE-1 hypomethylation was also detected in the white blood cells of cancer patients (Hsiung et al. 2007; Wilhelm et al. 2010). The source of the hypomethylated cells in cancer patients still needs to be identified to determine whether these cells are from cancer cells or from normal cells with systemic hypomethylated LINE-1s. Nevertheless, this evidence suggests that LINE-1 methylation is a promising marker in cancer risk prediction.

Cells must have a correct amount of LINE-1 methylation to maintain their physiological functions (Aporntewan et al. 2011). Consequently, there is a wide range of LINE-1 methylation levels found in normal cells, depending on cell type (Chalitchagorn et al. 2004). This methylation range leads to low specificity when using LINE-1 hypomethylation as a cancer screening marker. The ability to distinguish between normal and tumor DNA is low, particularly because clinical samples, including plasma, mouth washes, or Papanicolaou smears, are routinely contaminated with DNA from several normal cell types. LINE-1 methylation pattern analysis demonstrated unprecedented characteristics of LINE-1 partial methylation in normal cells and in the cancer global hypomethylation process (Pobsook et al. 2011). The interchangeable pattern between LINE-1 hypermethylation and partial methylation is a mechanism that may result in different LINE-1 methylation levels in normal cells (Pobsook et al. 2011). In cancer, global hypomethylation is observed because of the loss of methylation of previously hypermethylated and partial methylated loci. Most PCR-based LINE-1 methylation measurement techniques cannot differentiate unmethylated CpG dinucleotides of partially methylated LINE-1s from unmethylated LINE-1s. There was a recent report using COBRA to classify LINE-1s into the three classes. This report showed that the number of unmethylated LINE-1 loci was a more sensitive and specific marker than LINE-1 methylation level to detect cancer DNA in mouthwash samples (Pobsook et al. 2011). It may be interesting to compare the number of unmethylated LINE-1 loci with LINE-1 methylation levels in other clinical samples. Moreover, it may be worth exploring whether changes in partially methylated LINE-1 loci can be observed in, and are able to predict, malignant transformation in pathological lesions in the very early stages of carcinogenesis or tissues in patients at risk of developing cancer.

Although the methylation of a majority of LINE-1 loci is reduced in cancer, some loci are unchanged. Currently, there are several advanced genomic techniques, including deep sequencing (Xie et al. 2009; Xie et al. 2011) and custom-made microarrays (Szpakowski et al. 2009), that are capable of measuring the methylation level of each LINE-1 or IRS locus. These approaches identified certain classes of LINE-1s and IRSs that more frequently show loss of methylation in cancer. Improved deep sequencing techniques will be able to determine the proportions of the three LINE-1 methylation classes at each LINE-1 locus. It is important to reevaluate the clinical significance of LINE-1 methylation by these advanced techniques. These methods should help define the relevant LINE-1 locations, sequences, and methylation patterns that are specific to carcinogenesis. Moreover, some intragenic LINE-1 loci are methylated *cis*-regulatory elements of their host genes (Aporntewan et al. 2011). Altered expression of these genes may lead to certain cellular phenotypes and clinical presentations. Genome-wide arrays or deep sequencing may be used to design promising new sets of methylated LINE-1 PCR-based techniques specifically aimed for the classification of the epigenome of the tumor phenotype.

Interestingly, some pathological lesions with increased potential for malignant transformation, such as myelodysplastic syndrome lesions, liver cirrhosis, and partial hydatidiform moles, possess LINE-1 hypermethylation (Takai et al. 2000; Romermann et al. 2007; Perrin et al. 2007). Further descriptive studies of other lesions, genomic distributions, and methylation patterns will clarify in detail whether this epigenetic process occurs during the early steps of LINE-1 hypomethylation in cancer. It is important to note that genome-wide hypomethylation in cancer can result in hypermethylated LINE-1s at some loci (Fig. 4; Phokaew et al. 2008). If LINE-1 hypermethylation and hypomethylation are present at the same loci in premalignant tissues and cancer, this finding would be a breakthrough by showing that epigenomic changes precede genetic changes during carcinogenesis. Detailed molecular biological approaches to explain how LINE-1 methylation fluctuates from hypermethylation to hypomethylation will be important to understand the development of global hypomethylation in cancer.

Finally, global hypomethylation mechanisms may be crucial for future cancer prevention and treatment. Genome-wide hypomethylation is common, occurs at an earlier stage of carcinogenesis, and is still an active process in most cancers (Tables 1 and 2 and ESM Table 1). Global hypomethylation is an epigenomic process that leads to cellular phenotypic changes. LINE-1 hypomethylation in cancer alters the expression of a large number of genes. Therefore, this epigenomic alteration should be an important target for future cancer prevention strategies. More-

over, unlike mutation, hypomethylation is reversible. Therefore, global hypomethylation in cancer is a candidate for new cancer treatments in the future.

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